

# Antiplatelet effect of aspirin in patients with coronary artery disease

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## PREFACE

Cardiovascular disease (CVD) is the number one cause of death globally, and atherothrombosis is the underlying cause of most cardiovascular events [1,2]. Platelet-dependent thrombus formation on pre-existing atherosclerotic plaques constitutes the most important pathophysiological mechanism, and for several years the platelet function inhibitor aspirin (acetylsalicylic acid) has been a cornerstone in the prevention and treatment of CVD [2,3]. Coronary artery disease (CAD) and stroke are the most frequent manifestations of CVD, which remains the leading cause of death according to the World Health Organisation (WHO) [1]. Despite improved primary prevention strategies and a growing armamentarium of antiplatelet drugs, the WHO expects the total number of CVD-related deaths to rise considerably, mainly due to an increasing incidence of the disease [3,4]. It is therefore important to increase our understanding of the underlying pathophysiology and develop new therapeutic strategies. This thesis evaluates and compares a number of platelet function tests and explores platelet turnover as a potential mechanism of reduced antiplatelet effect of aspirin in patients with CAD.

## LIST OF PAPERS

The thesis is based on four separate studies (studies 1–4) and the following four papers:

**Paper I:** A comparison of platelet function tests and thromboxane metabolites to evaluate aspirin response in healthy individuals and patients with coronary artery disease. Grove EL, Hvas AM, Johnsen HL, Hedegaard SS, Pedersen SB, Mortensen J, Kristensen SD. *Thromb Haemost* 2010;103:1245-1253.

**Paper II:** Immature platelets in patients with acute coronary syndromes. Grove EL, Hvas AM, Kristensen SD. *Thromb Haemost* 2009;101:151-156.

**Paper III:** Effect of platelet turnover on whole blood platelet aggregation in patients with coronary artery disease. Grove EL, Hvas AM, Mortensen SB, Larsen SB, Kristensen SD. *J Thromb Haemost*. 2011; 9:185-191.

**Paper IV:** Patients with previous definite stent thrombosis have a reduced antiplatelet effect of aspirin and a larger fraction of immature platelets. \*Würtz M, \*Grove EL, Wulff LN, Kaltoft AK, Tilsted HH, Jensen LO, Hvas AM, Kristensen SD. *J. Am. Coll. Cardiol. Interv.* 2010; 3:828-835. \*Shared first authorship.

## LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
ARU	Aspirin reaction units
CAD	Coronary artery disease
COX	Cyclooxygenase
CV	Coefficient of variation
CVD	Cardiovascular disease
ELISA	Enzyme-Linked Immunosorbent Assay
DNA	Deoxyribonucleic acid
GP	Glycoprotein
IPC	Immature platelet count
IPF	Immature platelet fraction
LTA	Light transmission aggregometry
PCI	Percutaneous coronary intervention
PDW	Platelet distribution width
PFA	Platelet function analyser
P-LCR	Platelet large cell ratio
PPIs	Proton pump inhibitors
MEA	Multiple electrode aggregometry
MI	Myocardial infarction
MPV	Mean platelet volume
NSAIDs	Non-steroidal anti-inflammatory drugs
RNA	Ribonucleic acid
RPR	Residual platelet reactivity
sP-selectin	Soluble P-selectin
ST	Stent thrombosis
STEMI	ST-segment elevation myocardial infarction
S-TXB <sub>2</sub>	Serum thromboxane B <sub>2</sub>
Thromboxane	TX
WHO	World Health Organisation

## INTRODUCTION

### PLATELETS

Platelets, or thrombocytes (from Greek θρόμβος – ‘clot’ and κύτος – ‘cell’), are the smallest of circulating blood cells, averaging only 2 to 5 µm in diameter and 0.5 µm in thickness, yet they have a key role in normal haemostasis and are important contributors to atherothrombotic disease. Furthermore, it is increasingly recognised that platelets, in addition to their haemostatic function, also have less well-understood roles in inflammation, atherosclerosis, tumour growth, metastasis, and angiogenesis [5]. The concentration of platelets in the blood normally ranges from 150 to 400 x 10<sup>9</sup>/L, thus far exceeding what is needed to secure haemostasis [6]. In the circulating blood, platelets are pushed to the vessel wall by blood flow, thus putting them close to the luminal surface of the endothelium and in an optimal position to rapidly detect and respond to vascular injury. Among many unique features, platelets hold the ability to change shape from the resting discoid form to their active shapes as described in more detail below. The platelet plasma membrane has a thick external coating, glycocalyx, but the lipid bilayer on which the glycocalyx rests is a typical unit membrane. Importantly, embedded in the membrane and present in storage granule membranes are several glycoprotein receptors, some of which (e.g. P-selectin, see ‘Methods’) are only expressed on the surface after activation. These receptors are of paramount importance to the haemostatic and thrombotic potential of platelets; they act as adhesion molecules, activate platelets, and contribute to both positive and negative regulatory feedback loops. Platelets are anucleate and are therefore, by definition, not real cells, although most often referred to as such. Since platelets are anucleate, they generally have a limited biosynthetic capacity to enable *de novo* synthesis of membrane and secretory proteins. However, newly formed platelets differ from other platelets.

### PLATELET PRODUCTION

Under steady-state conditions and an approximate platelet life-span of 8 to 10 days, humans must produce at least  $1 \times 10^{11}$  platelets each day to maintain a normal platelet count, and this level of production even increases under conditions of increased demand. Platelets are formed from megakaryocytes, which are highly specialised precursor cells with the sole function of releasing platelets into the circulation. Like other blood cells, megakaryocytes develop from haematopoietic stem cells residing in the bone marrow [7]. By the unique process of endomitosis, megakaryocytes undergo multiple replications of deoxyribonucleic acid (DNA) without cell divisions, resulting in giant cells. Upon endomitosis, polyploid megakaryocytes begin an expansion phase characterised by the production of proteins and granules essential for platelet function and the formation of an elaborate system of invaginated plasma membranes, which organise cytoplasmic organelles into domains representing nascent platelets [6,8]. The final stages of platelet formation are not fully understood, but involve cytoplasmic fragmentation of megakaryocytes and detachment of pseudopodia-like extensions termed proplatelets [7,9]. In the bone marrow, megakaryocytes are located in close proximity to the sinusoidal walls, thus facilitating the release of large cytoplasmic segments into the peripheral blood. As a result of the shear forces of circulating blood, these segments are fragmented into individual platelets [10].

Megakaryocyte development and platelet formation are regulated at multiple levels by many cytokines, including thrombopoietin, stromal cell-derived factor 1, Interleukin-3, 6, and 11, and stem cell factor, also known as steel factor or kit-ligand.

Thrombopoietin is the principal regulator of thrombopoiesis and regulates all stages of megakaryocyte development [7,9,11,12]. The binding of thrombopoietin to its megakaryocyte receptor inhibits apoptosis and results in an increased number, size, ploidy, and rate of maturation of the megakaryocytes [12].

### PLATELET TURNOVER

The number and fraction of newly released platelets reflects thrombopoiesis and the rate of platelet turnover, because the number of immature, reticulated platelets released from the bone marrow is increased in individuals with a high platelet turnover, including thrombocytopenic patients with increased peripheral platelet destruction [13-18]. Therefore, quantification of this platelet subpopulation can be used as a proxy for platelet turnover.

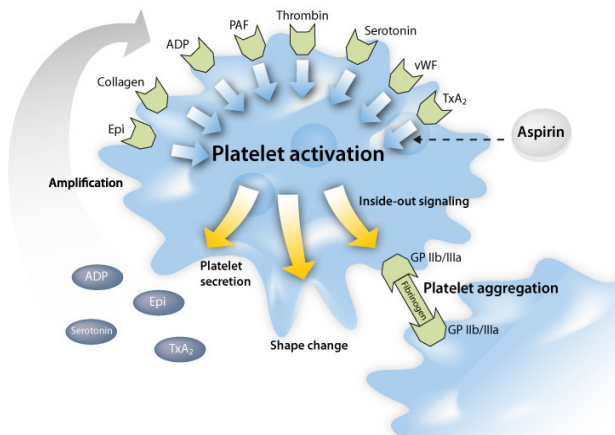
In 1969, the platelet analogue for red cell reticulocytes, ‘reticulated’ platelets, were first described by Ingram and Coopersmith who reported that newly formed platelets contain ribonucleic acid (RNA), which is stainable with methylene blue and has a characteristic appearance when a blood smear is observed using light microscopy [19]. In a study on beagle dogs, they showed that after acute blood loss, a unique population of platelets remarkable for their large volume and increased density appeared in the peripheral blood. This platelet subpopulation was identified by their tinctorial characteristics after the incubation of whole blood according to the routine procedure for demonstrating reticulated erythrocytes, and these cells were therefore referred to as ‘reticulated’ platelets [19]. These newly formed platelets were present in greatest numbers 5 to 10 days after blood loss, which coincided with the period of raised platelet counts. As described in more detail below (section on ‘Methods’), newly formed platelets can also be identified using RNA fluorescent dyes and flow cytometry. In the literature, the terms ‘reticulated’ and ‘immature’ are often used interchangeably [16,20], although ‘immature’ platelets may be a more representative term [16].

The RNA in immature platelets is mainly of ‘messenger’ type (mRNA), and together with small amounts of rough endoplasmic reticulum and ribosomes, originates from the megakaryocyte extensions formed during the late phase of maturation [21]. Although the mRNA is unstable and degrades within approximately 24 hours in the circulation [22,23], it enables newly formed platelets to synthesise proteins despite the lack of a nucleus. Accordingly, the production of major membrane glycoprotein (GP)s, enzymes, and alpha-granule proteins have been reported and include thrombosis-related proteins such as fibrinogen, von Willebrand factor, P-selectin, GP IIb/IIIa (αIIbβ3), and cyclooxygenase (COX)-2 [24,25]. As discussed below, these platelet proteins may enhance the haemostatic potential of platelets [20,24,26].

### PLATELET AGGREGATION AND CORONARY THROMBOSIS

When platelets are activated inappropriately, they are also important contributors to the development of atherothrombotic disorders, including stent thrombosis (ST) [27] and acute coronary syndromes (ACS), with clinical presentations covering unstable angina, non-ST-segment myocardial infarction (non-STEMI), STEMI, and sudden cardiac death. The central role of coronary thrombosis in ACS has been demonstrated by angiographic and optical coherence tomography detection of arterial thrombi at the site of the culprit lesion [28-30] and by means of autopsy data [31,32]. Thrombosis most often develops at sites of vulnerable plaques characterised by a high concentration of inflammatory

cells, low density of smooth muscle cells and a large lipid-rich core covered by a thin fibrous cap [33,34].



**Figure 1.** Platelet activation and aggregation. Epi: epinephrine; ADP: adenosine diphosphate; PAF: platelet-activating factor; vWF: von Willebrand Factor;  $TxA_2$ : thromboxane  $A_2$ ; GP: glycoprotein.

A seminal event in the pathophysiology of ACS is plaque rupture or erosion that exposes collagen, von Willebrand factor, and other subendothelial agents, sharing the ability to bind and activate platelets. The quantitative contribution of these and other platelet-activating pathways is partially dependent on the amount of physical shear stress applied by the blood flow, and shear stress per se also activates platelets [35]. Upon activation, platelets change from the normal disc shape to a compact sphere with long dendritic extensions facilitating adhesion and aggregation [6]. Furthermore, they release thromboxane ( $Tx A_2$ ), epinephrine, serotonin, and ADP, which, acting in synergy with thrombin produced by the coagulation cascade, amplify platelet activation (Figure 1). During platelet activation, calcium translocation within the platelet plays a central role as it increases the release of ADP from the dense granules, causing an amplification of platelet activation by autocrine and paracrine stimulation of P2Y1 and P2Y12 ADP-receptors [36]. Calcium translocation also induces phospholipase A2 activation that triggers the metabolism of arachidonic acid (AA) and, as described in more detail below, ultimately results in the production of  $TxA_2$ , a vasoconstrictor and platelet agonist. Additionally, irrespective of the type of agonist, calcium promotes inside-out signalling and conveys a modulatory change in the avidity and affinity of GP IIb/IIIa receptors, often termed the final common pathway of platelet aggregation [37]. This receptor enables the formation of platelet-platelet interactions needed for thrombus growth to occur. The conformational change induced by platelet agonists thus increases the receptor affinity for von Willebrand factor and fibrinogen, which both have several GP IIb/IIIa binding sites and are thus able to bridge platelets to the vessel wall or to each other (Figure 1). With at least 50,000 GP IIb/IIIa receptors on the surface, platelets aggregate into a three-dimensional haemostatic plug stabilised by fibrin, the end product of the coagulation cascade. The development of the platelet-rich thrombus on atherosclerotic plaques is further promoted by the fact that dysfunctional endothelial cells fail to produce platelet antagonists such as prostacyclin and nitric oxide, which are normally constitutively produced by the endothelium [38].

In summary, formation of the platelet plug occurs in three stages: adhesion of platelets to the vessel wall, amplification of platelet activation, and, finally, platelet aggregation. Acknowledg-

ing the crucial role of platelets, strategies to treat and prevent arterial thrombosis include the widespread use of platelet function inhibitors such as aspirin.

#### ASPIRIN: A SHORT SUMMARY OF A LONG HISTORY

The fascinating history of aspirin and aspirin-like remedies can be traced back to antiquity, when decoctions of willow bark were known for their antipyretic and analgesic effects. In 1828, the active extract of the bark (salicin, after the Latin name for White Willow – *Salix alba*) was isolated in crystalline form by Henri Leroux, a French pharmacist. Ten years later an Italian chemist named Raffaele Piria succeeded in separating out the acid in its pure state. However, it was not until 1897 that Felix Hoffmann, a chemist at Bayer's laboratories, synthesised the acetylated form of salicylic acid, which was marketed in 1899 under the name of Aspirin (A for acetyl, and spir for spiric acid, the former name of salicylic acid). Ironically, the Bayer company, which initially promoted Aspirin as a painkiller, issued a reassurance to the public that 'Aspirin does not affect the heart', a statement which was later substituted with the slogan 'An Aspirin a day helps keep heart attack away'.

The antithrombotic properties of aspirin were first reported in 1953 [39] by Lawrence Craven, a general practitioner in Glendale, California. Dr Craven noticed that patients who took Aspergum (an analgesic aspirin-containing gum) e.g. after tonsillectomy or tooth extraction, had a tendency to bleed more easily. The pathogenesis of myocardial infarction (MI) and the effects of aspirin were incompletely understood at the time, but Craven successfully tested his hypothesis that aspirin would prevent MI [39,40]. The mechanism conferring the cardioprotective effect of aspirin was later elucidated by Weiss and Aledort who reported the antiplatelet effect of aspirin [41] and by Nobel Prize laureate Sir John Vane who demonstrated that the main mechanism of action was the inhibition of prostaglandin synthesis [42,43]. Now, more than a century after its commercialisation, aspirin remains one of the most widely used drugs in the world.

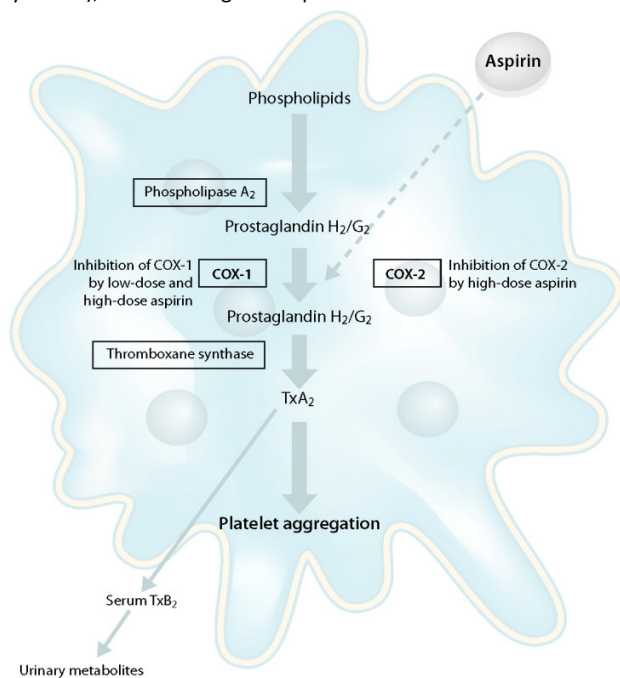
#### PHARMACOLOGY OF ASPIRIN

Aspirin's mechanisms of action and associated kinetics have been studied intensively, mainly in healthy individuals. Aspirin is rapidly absorbed in the stomach and upper small intestine with plasma levels peaking 30 to 40 minutes after ingestion of non-enteric coated preparations. Over a wide range of doses, the oral bioavailability of non-enteric coated aspirin is 40 to 50% [44], whereas the bioavailability of enteric-coated tablets and sustained-release preparations is significantly lower [45]. The half-life of aspirin in plasma is only 15 to 20 minutes, yet the platelet inhibitory effect lasts for the 8 to 10 day platelet lifespan owing to the irreversible inactivation of the COX-enzyme [46,47]. Platelets are anucleate, and COX regeneration is thus not possible. Therefore, the platelet inhibitory effect of aspirin is reversed only through the generation of new platelets, thus allowing the use of a once-daily regimen despite the short half-life of the drug [48].

In endothelial cells the production of prostacyclin is decreased by aspirin, but despite the vasodilatory and platelet inhibitory effects of prostacyclin, platelet inhibition remains the prevailing effect of aspirin. This is explained by the permanent COX-inactivation in the anucleate platelet, which contrasts with the ability of endothelial cells to retain their capacity to produce new COX. Furthermore, aspirin acetylates platelets in the portal vascular system before reaching the general circulation. As it passes the liver, up to 50% of the drug is metabolised, and the concentration of active drug is further diluted when joining the

rest of the venous blood [43]. In addition to the inactivation in the liver, aspirin is metabolised by esterases in the blood and the gastrointestinal mucosa. Salicylates are excreted mainly by the kidneys, with salicyluric acid being the predominant metabolite.

Treatment with daily low-dose aspirin (i.e. doses in the range of 75 to 325 mg) primarily inhibits platelet aggregation by effectively reducing the production of  $\text{TXA}_2$ , a vasoconstrictor and platelet activator. It has previously been shown by our and other groups that even low-dose aspirin reduces thromboxane production almost completely as indicated by a > 98% inhibition of serum thromboxane  $\text{B}_2$  [49-51]. As discussed in more detail below (Methods: 'Measuring the antiplatelet effect of aspirin'),  $\text{TXB}_2$  is metabolised by two major pathways, resulting in the formation of 2,3-dinor- $\text{TXB}_2$  and 11-dehydro- $\text{TXB}_2$ , both of which circulate in the plasma and are excreted in the urine.  $\text{TXA}_2$  normally induces platelet activation that is amplified through release of ADP and consequent ADP-induced platelet activation, as described above (Figure 1). Since  $\text{TXA}_2$  is synthesised and released by platelets in response to many stimuli, including thrombin, collagen and ADP, it provides a mechanism for amplifying the platelet response to several agonists [47,52].  $\text{TXA}_2$  is formed after phospholipase-mediated release of AA from phospholipids in the platelet cell membrane, as depicted in Figure 2. The enzyme thromboxane synthase produces  $\text{TXA}_2$  from prostaglandin  $\text{H}_2$ , which is formed from AA by the COX enzyme (also referred to as prostaglandin H synthase), the main target of aspirin.



**Figure 2.** Pharmacology of aspirin. The names of enzymes are written in bold-italics. COX: cyclooxygenase; Tx: thromboxane.

There are two COX isoforms, COX-1 and COX-2. COX-1 is considered a constitutive enzyme and is found in most mammalian cells, whereas COX-2 is an inducible enzyme undetectable in most tissues, but abundant in, e.g., activated macrophages and other inflammatory cells upon stimulation. Although COX-1 is often considered the only isoform in platelets [43,53], it has been shown that COX-2 is present during megakaryocytopoiesis and is expressed by megakaryocytes and newly formed platelets [24,54].

COX enzymes are inhibited by aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) most of which are non-selective and

inhibit all COX isoforms. The consequential inhibition of thromboxane and prostaglandin synthesis results in anti-inflammatory as well as antipyretic, analgesic, and antithrombotic effects [43]. As prostaglandins normally have a protective role in the gastrointestinal tract, the inhibition of prostaglandin synthesis explains why irritation of the gastric mucosa with dyspepsia is the most frequent adverse effect of NSAIDs [55]. Aspirin is a non-selective COX-inhibitor, yet low-dose aspirin primarily inhibits COX-1 [43,47,53]. The fact that higher doses are needed to inhibit COX-2 may partly explain why higher aspirin doses are needed to achieve anti-inflammatory and analgesic effects, whereas platelet inhibition and more than 95% inhibition of thromboxane production can be obtained using daily doses as low as 30 to 50 mg [48,56,57]. Theoretically, higher doses of aspirin or more frequent dosing may be needed when platelet turnover is increased. The aspirin-induced inhibition of COX-1 is achieved within 1 hour of oral administration and is mediated through irreversible acetylation of serine 529 [58]. This results in a conformational change in the active site of the enzyme and prevents the binding of AA. In contrast, most other NSAIDs such as ibuprofen produce reversible inhibition of COX by competing with AA for the active site of the enzyme.

The benefits of aspirin in preventing and treating atherothrombotic disease may not be mediated solely through inhibition of platelet thromboxane production, although this is undoubtedly the predominant mechanism of action. Other potential mechanisms are thought to be dose-dependent and include the reduced release of oxygen radicals, growth factors, and inflammatory cytokines [47,55] as well as anticoagulant properties and effects on fibrin clot structure and clotting factors [59-61].

#### CLINICAL USE OF ASPIRIN

Aspirin is a cornerstone in the prevention and treatment of CVD [2,3]. The safety and efficacy of the drug has been evaluated in several populations, ranging from apparently healthy individuals at low risk of suffering cardiovascular events to high-risk patients presenting with ACS or an acute ischaemic stroke. Numerous clinical trials have demonstrated that aspirin is effective for both primary and long-term secondary prevention of ACS and ischaemic stroke, reduces cardiovascular mortality, and is effective in the acute treatment of ACS and ischaemic stroke [62,63]. A detailed discussion of all clinical trials with aspirin is outside the scope of this thesis, and recent reviews have discussed clinical trials of antiplatelet drugs, including aspirin [3,64].

A comprehensive meta-analysis including a total of 287 studies showed that in patients at high risk of cardiovascular events due to acute or previous vascular disease or other predisposing conditions, long-term antiplatelet therapy reduces the risk of serious vascular events (nonfatal stroke, nonfatal MI, vascular death) by approximately one quarter; nonfatal MI was reduced by one third, nonfatal stroke by one quarter, and vascular mortality by one sixth [63]. Against this benefit must be weighed an increased risk of bleeding. Long-term therapy with low-dose aspirin approximately doubles the risk of major extracranial bleeding, corresponding to an estimated absolute excess of 1 to 2 major bleeding complications per 1000 middle-aged patients treated with low-dose aspirin for 1 year [65]. Furthermore, daily low-dose aspirin results in an absolute excess of 1 to 2 haemorrhagic strokes per 10,000 patients [63]. Thus, in the absence of increased susceptibility to bleeding, the number of high-risk patients avoiding a serious vascular event clearly outweighs the number with a major bleeding, and the overall benefit-risk ratio of low-dose aspirin in secondary prevention is favourable.

For primary prevention, however, the balance is less clear. A recent meta-analysis included six primary prevention trials with a total of 95,000 low-risk individuals [66]. Compared with control (placebo, vitamin E etc, but no other antiplatelet agents), low-dose aspirin (ranging from 100 mg on alternate days to 500 mg once daily) significantly reduced the relative risk of MI, stroke, or vascular death by only 12% (absolute risk reduction 0.07% per year). Importantly, this benefit was obtained at the expense of an increased risk of fatal haemorrhagic stroke (relative risk 1.73 [99% confidence interval 0.96–3.13],  $p = 0.02$ ) and an increased risk of major gastrointestinal and other extracranial bleeding complications (relative risk 1.54 [1.30–1.82],  $p < 0.0001$ ) [66]. No significant effect of low-dose aspirin was observed on vascular, non-vascular, or total mortality. As discussed by the authors, one may hypothesise that statin therapy may confer an equally effective primary prevention with fewer hazards (i.e. no increased risk of bleeding).

In conclusion, the favourable benefit-risk ratio of low-dose aspirin for secondary prevention is reflected in European and American clinical guidelines stating that low-dose aspirin should be used in high-risk patients, whereas the modest absolute cardiovascular risk reductions in low-risk individuals do not justify a routine use of aspirin [67-71]. For primary prevention, low-dose aspirin may be beneficial in certain subsets of patients, but the well-documented risk of bleeding should be carefully considered.

#### VARIABILITY IN THE ANTIPLATELET EFFECT OF ASPIRIN

Notwithstanding the cost-effective benefit of aspirin in the treatment of acute CVD and secondary CVD prevention, many patients experience cardiovascular events (often termed clinical low-responsiveness or treatment failure) despite daily use of low-dose aspirin. Aspirin thus fails to prevent a substantial number of recurrent serious vascular events among high-risk patients, and during a 2-year follow-up, one in eight patients experiences a recurrent vascular event [63,72]. This is not surprising, as several factors likely contribute to this residual risk of cardiovascular events. Firstly, the use of aspirin and other drugs recommended for secondary prevention is suboptimal as shown in a study on temporal trends in the use of evidence-based pharmacological and interventional therapies after ACS [73]. Secondly, as shown in Figure 1, aspirin blocks just one of several platelet-activating pathways, thus enabling platelet activation by, e.g., thrombin. Thirdly, revascularisation procedures are effective in improving myocardial perfusion and relieving symptoms, but do not interfere with platelet-dependent thrombosis as the underlying pathophysiological mechanism of most cardiovascular events. Fourthly, given the multifactorial nature of atherothrombosis, it is not surprising that the use of one single strategy does not completely prevent recurrent cardiovascular events. Finally, many studies have demonstrated a considerable variability in platelet function during treatment with aspirin (often termed biochemical low-responsiveness) [74-76], and high residual platelet reactivity (RPR) during treatment with aspirin seems to correlate with a poor clinical outcome [77,78].

There has been great interest in platelet function testing and antiplatelet drug variability, and the topic remains the subject of ongoing controversy. As discussed below, inter-individual variability in the antiplatelet effect of aspirin is likely to be explained by multiple mechanisms, many of which are not fully elucidated. Given the prevalence of atherothrombotic disease, exploring these mechanisms is important as they may provide new pharmacological targets with the potential of improving the treatment of cardiovascular disease. As previously discussed, these mecha-

nisms are probably multifactorial, representing an interplay of clinical, cellular, and genetic factors [3] (Discussion, Figure 13).

As discussed in more detail below ('Discussion'), the prevalence of aspirin low-responders is highly dependent on the choice of platelet function test and, importantly, the extent of compliance control. Aspirin low-responsiveness has been reported in up to 57% of patients, but the prevalence is much lower when the effect of aspirin is assessed in fully compliant patients by methods that directly evaluate the effect of aspirin on its pharmacological target [51,79-81].

That the phenomenon of a reduced response to aspirin is not merely explained by poor compliance and variability in platelet function testing has been shown in studies using administration of large oral aspirin doses, observed aspirin ingestion, or the in vitro addition of aspirin at suprapharmacological concentrations [82-87]. Neither strategy was able to fully eliminate the existence of (biochemically) low-responsive individuals.

In addition to seeking new therapeutic targets, attempts have been made to refine the current 'one size fits all' approach to antiplatelet therapy. To the clinician, the relevance of platelet function testing lies in its ability to predict cardiovascular events and potentially guide and optimise antiplatelet therapy [3]. However, before pursuing such a strategy, one needs to carefully assess the performance of the platelet function tests employed. In particular, reproducibility should be evaluated.

#### AIMS AND HYPOTHESES

The main purpose of this thesis was to evaluate and compare a number of platelet function tests and to explore platelet turnover as a potential mechanism of reduced antiplatelet effect of aspirin in patients with CAD.

Four separate studies were performed with the following specific aims and hypotheses:

##### Study 1

**Aims:** To evaluate the reproducibility and mutual concordance of several platelet function tests, including the historical reference method (LTA) and recently developed, widely used point-of-care tests. Furthermore, to evaluate the association between these tests and thromboxane metabolites in serum and urine as pharmacologically specific measures of aspirin-induced COX-inhibition.

**Hypothesis:** The reproducibility of whole blood assays is at least as good as the reproducibility for the classical reference method. There is a moderate agreement between tests.

##### Study 2

**Aims:** To evaluate the fraction of immature platelets as a proxy for platelet turnover in healthy individuals, patients with stable CAD, and patients with ACS.

**Hypothesis:** Platelet turnover is increased in patients with ACS.

##### Study 3

**Aims:** To investigate the impact of platelet turnover on the antiplatelet effect of aspirin in patients with stable CAD and to identify determinants of platelet turnover.

**Hypothesis:** The antiplatelet effect of aspirin is reduced in patients with increased platelet turnover.

#### Study 4

**Aims:** To evaluate platelet turnover and the antiplatelet effect of aspirin in patients with angiographically verified ST compared with matched controls with no history of ST.

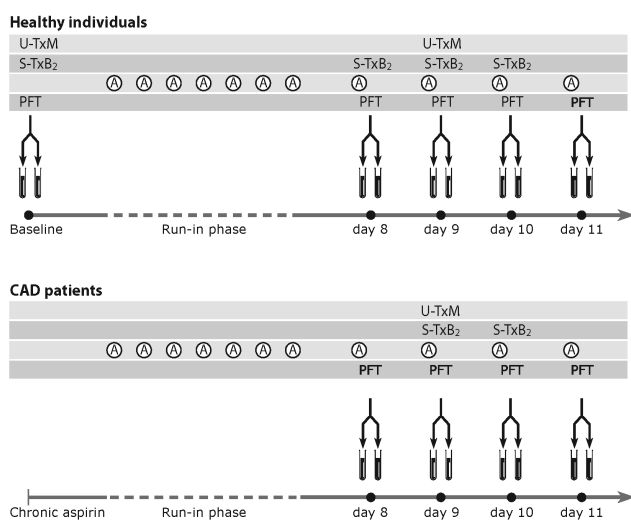
**Hypothesis:** The antiplatelet effect of aspirin is reduced in patients with previous ST.

#### METHODS

##### STUDY POPULATION AND DESIGN

A detailed description of study designs, inclusion and exclusion criteria is provided in papers I–IV. All patients were enrolled at the Department of Cardiology, Aarhus University Hospital, Skejby. Patients in studies 1, 3, and 4 were invited to participate upon identification in the Western Denmark Heart Registry, which collects patient- and procedure-specific information on coronary interventions [88].

**Study 1:** In this prospective, interventional study, we included 21 healthy individuals and 43 patients with stable CAD. Healthy individuals were mainly included from hospital staff. Baseline samples from healthy individuals were obtained prior to aspirin treatment. As depicted in Figure 3, all study participants were treated with 75 mg of non-enteric coated aspirin daily for 1 week prior to blood sampling for 4 consecutive days during continued aspirin treatment. Standardised blood sampling was performed at the same time of day for each participant, exactly 1 hour after aspirin intake.



**Figure 3.** Design of study 1. At baseline, platelet function testing (PFT) was performed in healthy individuals. All study participants were then treated with 75 mg of non-enteric coated aspirin daily for 1 week (dotted line) prior to blood sampling for 4 consecutive days during continued aspirin treatment. All patients were on chronic aspirin, and the run-in phase was performed in an attempt to optimise pharmacokinetics. All platelet function tests were performed in duplicate within 2 hours after sampling. Urinary thromboxane metabolites (U-TxM) and serum thromboxane B<sub>2</sub> (S-TxB<sub>2</sub>) were measured as indicated. Figure modified from Grove et al [89].

**Study 2:** In this observational study, healthy individuals and CAD patients from study 1 served as control groups for comparison with ACS patients. We consecutively enrolled patients admitted with acute chest pains suggestive of ACS. Based on cardiac markers and electrocardiographic changes as described in paper II, patients were separated into two groups: unstable angina/non-STEMI (n = 182) and STEMI (n = 177).

**Study 3:** In this observational study, we included 177 stable CAD patients on aspirin monotherapy. Patients with recent cardiovascular events were excluded to avoid dual antiplatelet treatment with clopidogrel in addition to aspirin, as this would affect platelet function measurements. Despite the exclusion of patients with recent events, the study population was not a low-risk population; it included a high prevalence of type 2 diabetics (48%) and patients with previous percutaneous coronary intervention (PCI) (92%), myocardial infarction (66%), coronary artery bypass grafting (23%), and stroke (10%).

**Study 4:** This study was a nested case-control study in 117 patients previously undergoing PCI. The study population included 39 patients previously diagnosed with definite ST according to the Academic Research Consortium criteria [90], and 78 patients with no history of ST serving as controls. Cases and controls were matched at a 1:2 ratio with respect to the following risk factors for ST: age, sex, stent type, and indication for PCI. A detailed description of the inclusion of ST patients is provided in the flow-chart in Figure 1, paper IV.

#### OPTIMISING COMPLIANCE AND PHARMACOKINETICS

As previously discussed, it is obviously of paramount importance to optimise compliance when investigating the pharmacological effect of a drug [75,91]. In studies 1, 3, and 4 we aimed at investigating the antiplatelet effect of aspirin, and it was therefore important to ensure that all study participants were fully compliant. Furthermore, although all patients were on chronic aspirin treatment, we wanted to optimise and make uniform the pharmacokinetics in these studies because not all patients took the same dose or preparation of aspirin.

Accordingly, all patients received a pill-dispensing box with seven tablets of non-enteric coated aspirin 75 mg (Hjerdyl®; Sandoz, Copenhagen, Denmark) in separate compartments for each day of the week. Patients were thoroughly instructed to save these for the last 7 days before blood sampling. Standardised blood sampling was performed exactly 1 hour after ingestion of aspirin. Contrary to most previous studies, we standardised this time interval to reduce the inter-individual variability in platelet aggregation. It has previously been shown that the number of non-aspirinated platelets differs between the early and late parts of the usual 24-hour dosing interval [50], and the importance of standardising the time interval between aspirin ingestion and blood sampling has recently been stressed in a study investigating the time-dependent efficacy of aspirin as evaluated by light transmission aggregometry (LTA) and serum thromboxane [92]. Finally, compliance was further optimised by face-to-face interviews and pill counting and was confirmed by measurements of serum TXB<sub>2</sub>, which is very sensitive to aspirin as is shown by a > 98% inhibition during treatment with low-dose aspirin [49-51]. As discussed in more detail below, serum TXB<sub>2</sub> is therefore considered the pharmacologically (i.e. biochemically) most specific test to evaluate the antiplatelet effect of aspirin [51,76,93].

Inter-individual variability in platelet aggregation was further reduced by standardised procedures for blood sampling as described below.

#### BLOOD SAMPLING AND ANTICOAGULANTS FOR ANALYSES OF PLATELET FUNCTION

Samples were collected from an antecubital vein using a large bore needle (19-G) and a minimum of stasis with patients in the supine position after 30 minutes of rest in the sitting position [94]. To minimise spontaneous platelet activation, the first millilitres of blood were collected in tubes that were not used for platelet aggregometry. All platelet function tests were performed within 2 hours after blood sampling. Additionally, in study 1 when evaluating day-to-day variation, platelet function tests were performed at the same time of the day for each study participant to avoid the potential influence of diurnal variations in platelet activity [95-97].

A detailed description of all anticoagulants used for analyses of platelet function and other parameters is provided in papers I–IV. Platelet function testing *in vitro* has many inherent problems, including the need for anticoagulants that may create an unphysiological milieu and entails a risk of affecting platelet aggregation. In our studies, citrated blood samples were employed for analyses of platelet aggregation. Citrate, which remains the most widely used anticoagulant for platelet function testing, acts by chelating extracellular calcium and may, therefore, influence platelet function to some extent, because divalent cations are important for several aspects of platelet function [98].

Accordingly, the use of other anticoagulants, such as unfractionated or low-molecular-weight heparin, melagatran, lepirudin, argatroban, and hirudin have been investigated in the literature [98,99]. In our most recent studies, including study 4, hirudin, a highly selective thrombin inhibitor, which does not affect ionised calcium levels, was used in addition to citrated samples in order to investigate the importance of anticoagulants on platelet function.

#### MEASURING THE ANTIPLATELET EFFECT OF ASPIRIN

Both acetylsalicylic acid (aspirin) and salicylic acid can be measured in plasma [100], but due to the short half-life, especially of the former, the antiplatelet effect of aspirin is instead more often evaluated by measuring COX-related metabolites or platelet function. Since AA is the substrate for COX-1, measurements of AA-induced platelet aggregation and TXB<sub>2</sub>-metabolites (Figure 2) are often employed. Aspirin intake should result in 1) failure of AA to induce platelet aggregation above a certain level and 2) reduced levels of TXB<sub>2</sub>-metabolites. As the main goal of aspirin treatment is to inhibit platelet aggregation, platelet function testing may be considered the most clinically relevant approach, whereas measurements of thromboxane metabolites are the most pharmacologically specific approach. In our studies, both strategies were used.

#### COX-RELATED METABOLITES

Since aspirin exerts its antiplatelet effect by inhibiting the COX-1-related production of the platelet activator TXA<sub>2</sub>, evaluating TXA<sub>2</sub>-production is a logical approach when evaluating the antiplatelet effect of aspirin [101] (Figure 2). However, it has previously been shown that quantification of circulating or urinary prostaglandin metabolites represents a more reliable way of assessing the endogenous prostaglandin synthesis *in vivo* than does quantification of TXA<sub>2</sub> [101]. Methods that directly evaluate the capacity of platelets to synthesise TXA<sub>2</sub> are preferred for their pharmacologi-

cal specificity. Previously, measurements of urinary 2,3-dinor-TXB<sub>2</sub> were used [101,102], whereas measurements of serum TXB<sub>2</sub> and urinary 11-dehydro-TXB<sub>2</sub> are now more widely employed.

The capacity of platelets to synthesise TXA<sub>2</sub> is reflected by a stable metabolite, serum TXB<sub>2</sub> (Figure 2). Since other cells only marginally affect TXB<sub>2</sub>-biosynthesis, serum TXB<sub>2</sub> is considered the most specific test for assessing the pharmacological effect of aspirin on platelets [49,76,93,103,104]. As discussed above in the 'Introduction', serum TXB<sub>2</sub> levels are very sensitive to even low-dose aspirin and are therefore an optimal way of testing adherence to aspirin treatment [49-51,91,105]. Some authors have measured TXB<sub>2</sub> levels in plasma instead of serum, but plasma TXB<sub>2</sub> is not an optimal reflection of *in vivo* TXA<sub>2</sub>-production [53,74,75,106].

The urinary levels of the most abundant metabolite of TXB<sub>2</sub>, 11-dehydro-TXB<sub>2</sub>, represents a time-integrated index of TXA<sub>2</sub> biosynthesis *in vivo* [102]. This stable metabolite is not formed in the kidneys and its urinary concentration therefore reflects systemic TXA<sub>2</sub>-production. Although low-dose aspirin provides an irreversible and almost complete inhibition of COX-1, nucleated cells such as monocytes and vascular endothelial cells can regenerate the enzyme within the usual 24-hour dosing interval for aspirin, thus enabling production of TXA<sub>2</sub> either directly or indirectly by providing prostaglandin H<sub>2</sub> to platelets [107,108]. It has been calculated that about 30% of the urinary metabolite derives from extra-platelet sources, and this fraction likely increases under clinical conditions with increased levels of inflammation [93,109]. Therefore, this method is not highly specific for monitoring the aspirin-induced inhibition of platelet COX-1.

COX-related metabolites were measured in studies 1, 3, and 4. Urinary 11-dehydro-TXB<sub>2</sub> levels were determined by collaborators in Chieti, Italy. Briefly, urinary samples (40 mL) were collected (Figure 3) and stored at -20 °C until extraction and analysis of 11-dehydro-TXB<sub>2</sub> levels by chromatographic and radioimmunoassay techniques [102,110], as previously described in detail [111]. Upon analysis, results were normalised for urinary creatinine concentrations. The assay variability was 10%, and the detection limit was 2–5 pg/mL urine.

Measurements of serum TXB<sub>2</sub> were performed according to Patrono et al [111] with the modification that serum TXB<sub>2</sub> was measured with an Enzyme-Linked Immunosorbent Assay (ELISA) (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, blood was collected in glass tubes without anticoagulant and allowed to clot at 37°C for 1 hour to induce maximum platelet activation and production of TXB<sub>2</sub>. The tubes were centrifuged for 10 minutes at 2600 g, and the serum removed and stored at -80°C. For analysis, samples were thawed, diluted, and measured in duplicate at two dilutions. Samples with results outside the standard curve were re-analysed with appropriate dilutions.

#### PLATELET ACTIVATION

An evaluation of *in vivo* platelet activation can be performed by flow cytometric determination of platelet surface P-selectin, GP IIb/IIIa or platelet-monocyte aggregates or by measuring soluble platelet release markers such as platelet factor 4, beta-thromboglobulin, glycoprotein V, and P-selectin. Platelet surface P-selectin has previously been considered the gold standard marker of platelet activation, but circulating platelet-monocyte aggregates may provide a more sensitive marker of *in vivo* platelet activation [112]. Importantly, flow cytometry-dependent methods are more demanding than the assessment of platelet release markers, which can be performed using ELISA kits. Therefore, in studies 3 and 4, we employed such kit to obtain a meas-

ure of *in vivo* platelet activation by determining the levels of soluble P-selectin (sP-selectin).

Soluble P-selectin was measured according to manufacturer's instructions (R&D Systems Europe, Abingdon, UK). P-selectin (CD62P) is an adhesion molecule confined to the alpha-granule membranes of resting platelets and is only expressed on the platelet surface during and after platelet degranulation and secretion [113]. In addition to its role in platelet activation, the P-selectin molecule is also involved in platelet aggregation, platelet-rolling on the vascular endothelium, and interactions between platelets, monocytes, endothelial cells, and procoagulant microparticles [113,114]. A soluble form of P-selectin has been characterised [115], and both the proportion of platelets expressing surface P-selectin as well as sP-selectin levels are increased in patients with ACS compared with healthy individuals and patients with stable angina [116].

#### PLATELET AGGREGATION

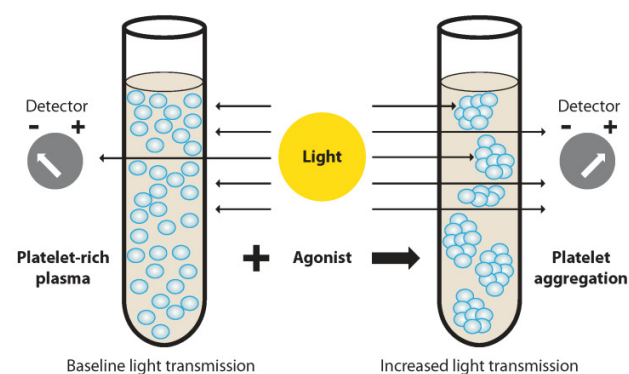
In studies 1, 3, and 4, a number of widely used platelet function tests were performed. In study 1 (Figure 3), the classical reference method, LTA, was compared with three point-of-care tests: the Platelet Function Analyser (PFA)-100®, VerifyNow®, and multiple electrode aggregometry (MEA, Multiplate®). The VerifyNow® and MEA were also employed in studies 3 and 4. The main advantages and drawbacks of individual tests are discussed below.

#### Light transmission aggregometry

Evaluation of platelet function is generally based on measurements of agonist-induced platelet aggregation. Historically, turbidometric LTA has been considered the 'gold standard' of platelet function tests, being the most widely used method to monitor the effect of aspirin and other antiplatelet drugs on platelet aggregation [117-121]. LTA, sometimes also referred to as optical platelet aggregometry, was first described by Gustav Born [122-124] and is based on the increase in light transmission through platelet-rich plasma as a result of agonist-induced platelet aggregation resulting in clump formation (Figure 4). Whole blood was slowly centrifuged (15 minutes at 100 g without brake) to sediment red and white cells, and the supernatant platelet-rich plasma was removed before the remaining blood was re-centrifuged (15 minutes at 1500 g with brake) to obtain platelet-poor plasma. The final platelet count in platelet-rich plasma was not adjusted [125]. The PAP-4D aggregometer (Bio/Data Corporation, Alpha Laboratories Ltd, Horsham, PA, USA) was adjusted to ensure that the difference in light transmission between platelet-rich and platelet-poor plasma was 100%. Results are given as the percentual change in light transmittance from baseline 5 minutes after addition of the agonist, using platelet-poor plasma as reference. Platelet aggregation was induced using a final agonist concentration of 1.0 mM AA (Medinova Scientific, Glostrup, Denmark). In accordance with previous studies, individuals with residual AA-induced platelet aggregation  $\geq 20\%$  were classified as aspirin low-responders [79,119,126-131].

Despite arbitrary definitions of sufficient platelet inhibition, positive correlations between impaired platelet inhibition and cardiovascular outcomes have been reported in several studies [127,132-136]. On the other hand, the performance of LTA is labour-intensive and time-consuming, thus limiting a broad-scale application in daily clinical practice. Recent efforts have been made to standardise the use of LTA, e.g. in patients with bleeding disorders [137], whereas no clear consensus exists on the performance of LTA to evaluate the antiplatelet effect of aspirin and other antiplatelet drugs. Furthermore, LTA is dependent on op-

erator and interpreter experience and is subject to many methodological variables, explaining the relatively poor reproducibility and lack of agreement with other platelet function tests reported by us and others [89,130,138]. As for all platelet function tests, physical activity, drugs, and diet (garlic, caffeine, polyunsaturated fatty acids etc) may influence platelet aggregation, and for LTA in particular, it must be remembered that platelets are very sensitive and can be readily activated during the preparation of platelet-rich plasma. This is primarily explained by the fact that centrifugation may modify platelet function and remove large platelets, which are more reactive than smaller platelets. Other possible causes of test variability include variations in temperature, pH, platelet count, fibrinogen concentration, and type of anticoagulant used [118,121,139,140]. As described in paper I and above ('Optimising compliance and pharmacokinetics'), several measures were taken to reduce the influence of these variables. Finally, LTA has the drawback of being non-physiological with regard to neglecting the assessment of interactions between platelets and other blood cells.



**Figure 4.** Light transmission aggregometry according to Born's technique. Platelet-rich plasma is stirred in a cuvette placed between a light source and a photocell. When an agonist is added, platelets aggregate and absorb less light, resulting in increased light transmission. Light transmission through platelet-poor plasma is defined as 100% and is used as reference.

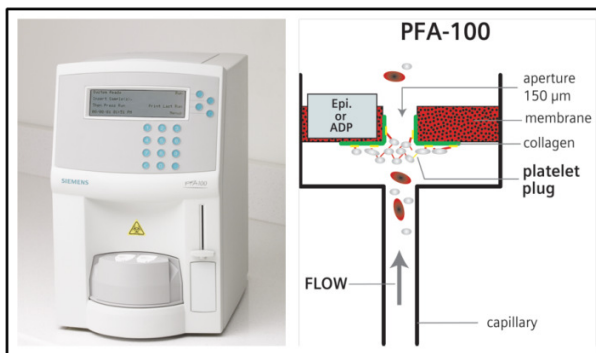
To overcome some of these limitations, a large number of bedside or 'point-of-care' platelet function tests have been developed, thus allowing simpler and more rapid assessments of platelet function. Additionally, these assays have the advantages of anticoagulated whole blood use (no need for sample preparation), usage of disposable cartridges or cups (no cleaning required), low sample volume, and no requirement for a skilled technician. These tests provide the possibility of widespread clinical use to evaluate platelet function in patients at risk of cardiovascular events and potentially guide and optimise antiplatelet therapy.

#### Platelet Function Analyser-100®

The PFA-100® (Siemens Healthcare Diagnostics, Marburg, Germany) measures platelet aggregation under high shear stress, mimicking flow conditions in a stenotic artery (Figure 5). The instrument aspirates citrated whole blood through a capillary and a microscopic aperture (147  $\mu\text{m}$ ) cut into a membrane coated with collagen and epinephrine. The presence of these platelet activators and the high shear rates (5000 to 6000  $\text{s}^{-1}$ ) under standardised flow conditions result in platelet adhesion, activation, and aggregation, thus building a stable platelet plug in the aperture [141]. The time required to occlude the aperture is



reported as closure time, and measurements are stopped when the aperture is occluded or after 5 minutes. The maximal closing time is therefore 300 seconds, and values higher than 300 seconds are reported as non-closure. A meta-analysis has shown that test results obtained with the PFA-100<sup>®</sup> significantly correlate with the risk of cardiovascular events [142]. The PFA-100<sup>®</sup> requires only a small volume of blood (800  $\mu$ L), is quick and easy to use, but test results might be affected by a number of variables including the haematocrit, platelet count, collagen platelet receptor density, and levels of von Willebrand factor [118,141]. Importantly, reproducibility of this assay is rather low, and one study reported that when using duplicate measurements, about 25 to 30% of samples would be classified differently with the two tests if a discrimination limit (normal vs. low-responder) for closure time of 170 or 190 seconds was used [143].



**Figure 5.** The Platelet Function Analyser-100<sup>®</sup> (from Siemens with permission).

#### **VerifyNow<sup>®</sup> Aspirin test**

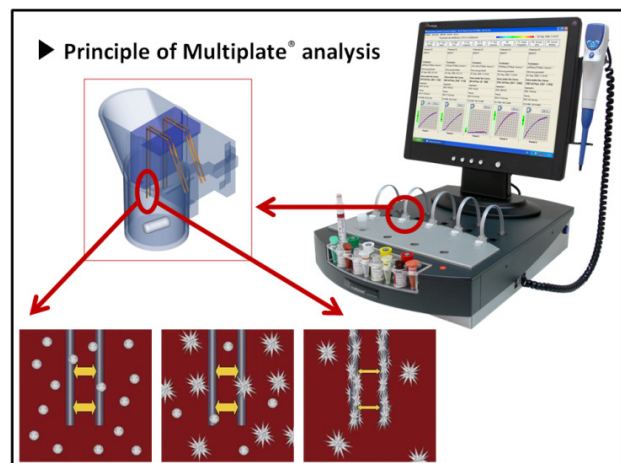
The VerifyNow<sup>®</sup> Aspirin test (Accumetrics, San Diego, CA, USA) is based on turbidimetric optical detection of platelet aggregation in whole blood (Figure 6). The instrument measures light transmittance (reported as Aspirin Reaction Units, ARU) through test cartridges with a mixing chamber containing fibrinogen-coated beads and platelet activators (metallic cations, propyl gallate, and AA) to stimulate the COX-1 pathway. Platelet aggregation is detected when activated platelets bind fibrinogen and agglutinate, thus increasing light transmission. Blood sample tubes are simply inserted onto the cartridge, which is premounted on the analyser. Samples are then analysed, and results are ready within a few minutes. From a research perspective, the prefabricated test cartridges are inflexible, but the fully automated test procedure enables widespread clinical use. A potential limitation of the analyser is the diagnostic limit (550 ARU) reported by the company. This limit was set in comparison with LTA in response to adrenaline in patients tested before and between 2 and 30 hours after a single 325-mg dose of aspirin [119].



**Figure 6.** The VerifyNow<sup>®</sup> device (from Accumetrics with permission).

#### **Multiple electrode aggregometry (Multiplate<sup>®</sup>)**

Multiple electrode aggregometry (Multiplate<sup>®</sup>; Dynabyte, Munich, Germany) is based on impedance measurements in whole blood (Figure 7). Aggregation was induced by addition of AA (Medinova Scientific, Glostrup, Denmark), ADP (Sigma-Aldrich, Broendby, Denmark), and collagen (Collagen Reagent Horm; Nycomed, Linz, Austria) at several concentrations, as described in papers I, III and IV. The use of such palette of platelet agonists at self-elected concentrations is possible owing to the computerised, five-channel analyser. The Multiplate<sup>®</sup> device has disposable cuvettes like the PFA-100<sup>®</sup> and the VerifyNow<sup>®</sup>. Each test cell contains two pairs of electrodes, thus enabling two simultaneous measurements. In study 1, true duplicate measurements were performed to evaluate the reproducibility of the analyser. Platelet aggregation was recorded for 6 minutes and reported as area under the curve (Aggregation Units x minutes), an integrated measure of aggregation velocity and maximal aggregation. From a research perspective the possibility of choosing individual agonists and concentrations is advantageous, whereas the need for incubation of blood (although at room temperature) for a minimum of 30 minutes and the need for manual pipetting limit a large-scale clinical implementation.



**Figure 7.** Multiple electrode aggregometry (Multiplate<sup>®</sup>) modified from www.multiplate.net with permission.

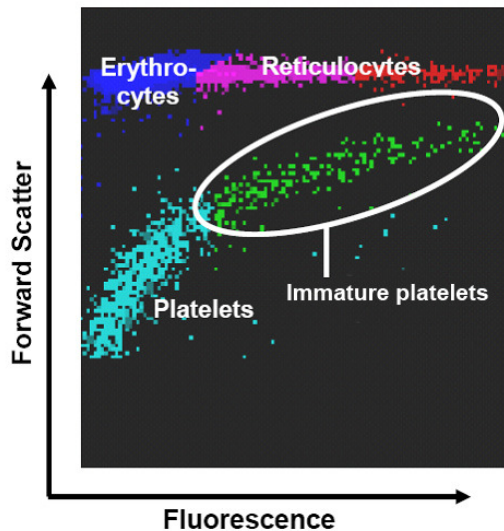
#### **PLATELET TURNOVER**

In studies 2, 3, and 4, platelet turnover was evaluated using automated flow cytometry with fluorescent RNA-staining dyes as described previously [16]. Determinants of platelet turnover were identified among baseline characteristics of study participants, and thrombopoietin, the main regulator of platelet production, was measured to further investigate platelet production and turnover.

#### **Platelet parameters**

Platelet characteristics and haematological parameters were obtained using an XE-2100 haematology analyser (Sysmex, Kobe, Japan), which has previously been described and evaluated in detail [144]. Peripheral blood samples were collected into EDTA (ethylenediaminetetraacetic acid dipotassium) and stored upright at room temperature until analysis, when samples were inverted 10 times as an automated procedure performed by the XE-2100. The analyser utilises automated flow cytometry and fluorescent dyes (polymethine and oxazine) that specifically label nucleic acids. Although young, RNA-containing platelets are primarily

stained, the whole platelet population is identified, thus providing the basis for optical platelet counts to be obtained in addition to impedance measurements. A switching algorithm chooses the most appropriate platelet count, which is determined by impedance in the majority (>95%) of samples. The switching algorithm comes into operation when there is an abnormality in the platelet volume distribution or when the platelet count is very low [145]. For example, in samples containing red cell fragments or large platelets, the optical (fluorescence) method is more accurate, whereas in the presence of white cell fragments, which are included in the optical count, the impedance count is automatically given priority [146].



**Figure 8.** Schematic scattergram showing immature platelets identified on the basis of forward scatter (cell volume) and fluorescence intensity (RNA content) (modified from Sysmex with permission).

As discussed above ('Introduction'), quantification of the immature platelet subpopulation can be used as a proxy for platelet turnover, since the number and fraction of newly released platelets reflects thrombopoiesis and the rate of platelet turnover. Differentiation between mature and immature platelets is performed with the above-mentioned flow cytometry software that has preset gates based on cell size (forward light scatter) and fluorescence intensity (RNA content) to discriminate between these platelet fractions (Figure 8). Absolute immature platelet counts (IPC) were obtained, and the immature platelet fraction (IPF) was calculated as the ratio of immature platelets to the total platelet count and is given in percent. The method demonstrates good reproducibility and stability in patients even after >24 hours of sample storage [16,147-149], and in a reproducibility study with 10 consecutive analyses performed in five healthy individuals, intra-assay coefficients of variation (CV) were on average 8.4% (5.3–12.0%, 'XE IPF master Reproducibility', provided by Sysmex).

Conventionally, immature reticulated platelets have been identified using manual flow cytometry after staining with a fluorescent dye such as thiazole orange [17]. Immature platelets can then be distinguished from mature platelets not taking up the dye. Initially, Kienast and Schmitz developed a flow cytometric analysis of platelet thiazole orange uptake in thrombocytopenic disorders [150]. This assay was later modified to include dual colour flow cytometry using thiazole orange and an anti-

glycoprotein Ib (CD42) monoclonal antibody conjugated to phycoerythrin [151]. However, due to difficulties in standardising this assay, it has not become a standard haematologic parameter for the estimation of thrombopoiesis and platelet turnover [152,153]. Potential sources of assay variability include sample preparation, temperature, non-specific as well as time- and concentration-dependent labelling with thiazole orange, and an absence of a standard against which to assess accuracy. These factors together with data analyses and interpretation of results explain the wide variation in normal ranges and inter-laboratory precision [16,17,147,152-154]. When using the new automated method (Sysmex), assay variability is likely reduced by the fully automated method comprising automated sampling, a fixed incubation time with RNA-staining dyes under strict temperature control, automatic scattergram analysis, and preset gates to discriminate between mature and immature platelets [155].

Unfortunately, the number of studies comparing the manual and the automated method is scarce and include only small populations of a variety of haematological disorders such as idiopathic thrombocytopenic purpura, aplastic anaemia, essential thrombocythaemia, and myelodysplastic syndrome [156,157]. The overall conclusion from these studies was that there is a good correlation between the two methods ( $r = 0.58-0.65$ ,  $p$ -values  $<0.01$ ) [156,157]. Importantly, when interpreting the results of these studies, the mixed study populations and the above-mentioned drawbacks of manual flow cytometry must be remembered, because this assay does not represent a gold standard for comparison. In addition to the flow cytometry-based methods, platelet kinetic parameters to evaluate platelet turnover can also be obtained using radioisotopic labelling for determination of platelet life span [158] and plasma glycofocalin measurements [159], although both methods suffer from several drawbacks [16].

Besides the use of absolute and relative immature platelet counts to evaluate platelet turnover, these parameters are increasingly used in haematology because they provide a non-invasive diagnostic tool for differentiating between consumptive and aplastic causes of thrombocytopenia and may also serve as a marker for predicting the timing of platelet recovery in cancer patients after chemotherapy and haematopoietic stem cell transplantation, thus potentially reducing unnecessary platelet transfusions [16,147,152,153,160].

Additional platelet parameters obtained from the XE-2100 analyser include the mean platelet volume (MPV), the platelet distribution width (PDW), and the platelet large cell ratio (P-LCR). These platelet size parameters are derived from the impedance platelet size distribution: MPV is calculated by dividing the platelet crit by the platelet count; PDW, a measure of platelet anisocytosis, is the width of the size distribution curve in femtolitres (fL) at the 20% level of the peak; P-LCR is the number of cells falling above the 12-fL threshold divided by the total number of platelets. The most important limitation of these platelet size parameters is the fact that platelet size increases over time in EDTA blood. For example, one study showed that over a 24-hour period, a 13% increase was seen in MPV, with the majority of this increase (11%) by 6 hours [161].

### Thrombopoietin

Thrombopoietin levels were determined using a commercial ELISA kit according to manufacturer's instructions (R&D Systems Europe, Abingdon UK). Whole blood was allowed to clot for 30 minutes at room temperature before serum was separated by centrifugation (1000 g for 15 minutes) and stored at -80°C until analysis.

## STATISTICAL ANALYSES

A detailed description of all statistical tests used in the studies is given in papers I–IV. Two-sided p-values <0.05 were considered statistically significant. When needed, statistical supervision was kindly provided by statisticians at the Departments of Biostatistics and Epidemiology, Aarhus University as acknowledged in the papers. Software packages STATA® version 10.0 (StataCorp, College Station, TX, USA) and GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA) were used for statistical analyses.

## SUMMARY OF RESULTS

The results of studies 1–4 are described in detail in the included papers. Below is given a summary of the results, including additional results from study 3. The summary does not include all statistics presented in the papers. For all analyses and comparisons, p-values, correlation coefficients, means, ranges etc. are available in the appended papers.

## COMPLIANCE

Patients were treated with aspirin in studies 1, 3, and 4. Pill counting and face-to-face interviews did not reveal any non-compliant individuals: all study participants claimed to be fully adherent to aspirin, and returned pill boxes were all empty. As shown in papers I, III, and IV, compliance was further confirmed by S-TXB<sub>2</sub> levels below a 10 ng/mL limit, which has previously been reported to reflect a more than 98% inhibition of platelet COX-1 activity [104].

## STUDY 1

This study showed that conclusions drawn from platelet function tests are highly dependent on the assay used: overall, reproducibility was moderate and the correlation between different tests was low.

The reproducibility of several platelet function tests was evaluated and showed that for all platelet function tests CVs were

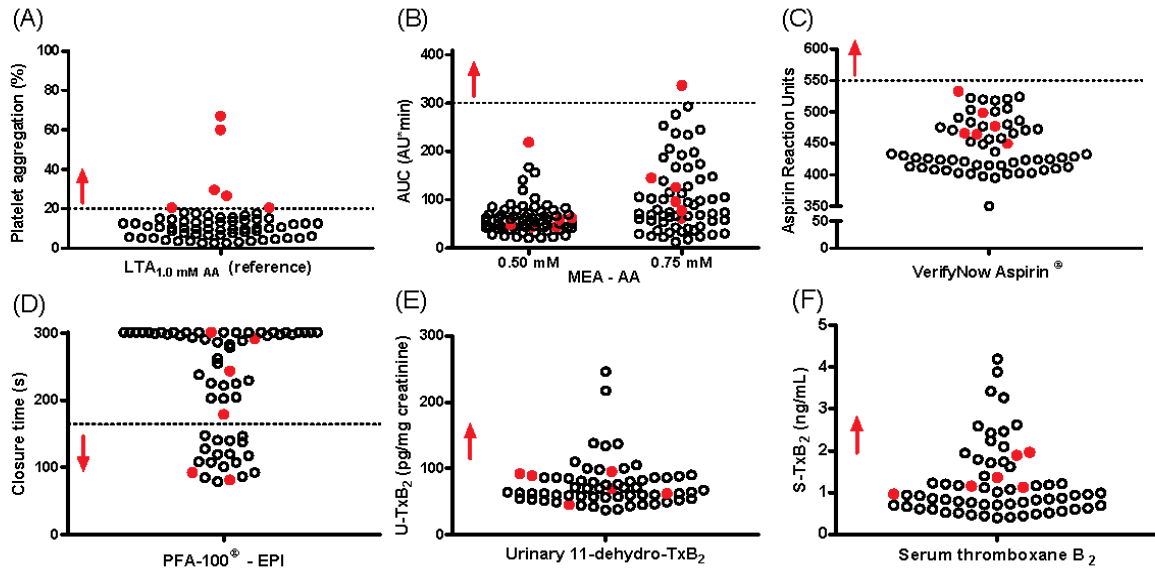
higher during aspirin treatment than at baseline (Table 1). When comparing individual assays, CVs were lowest for the VerifyNow® Aspirin test and highest for MEA at baseline as well as during aspirin treatment. CVs for day-to-day variation during aspirin treatment were also lowest for VerifyNow® Aspirin and high for MEA. However, day-to-day variation was equally high for AA-induced LTA, which is often considered the gold standard reference test [117–121].

As a measure of sensitivity for aspirin treatment, the ‘effect size’ of each platelet function test was calculated as previously reported [162] and explained in the table legend below (Table 1). According to these data, LTA and MEA seemed to be more sensitive for aspirin treatment than the VerifyNow® Aspirin test.

The agreement between the platelet function assays was carefully evaluated. In the literature, such comparisons are often based on cut-off levels to discriminate normal vs. low-responders, but, although these discrimination limits have been used by several studies, most cut-offs have not been carefully validated. Therefore, data were analysed using cut-off levels from the literature [79,119,126–131,163–165] as well as continuous variables of residual platelet aggregation by each platelet function test. When data were dichotomised into categorical variables, LTA with AA 1.0 mM as the agonist was chosen as reference test. In accordance with previous studies, participants with RPR ≥20% were considered aspirin low-responders. Using this cut-off, a total of six aspirin low-responders were identified with no difference between healthy individuals and CAD patients (p = 0.65). When RPR was defined according to MEA or the VerifyNow® Aspirin test, no study participants were classified as low-responders, whereas two study participants were classified as low-responders according to the PFA-100®. The agreement between tests was low (kappa ≤ 0.21 for all comparisons, paper I: Table 4), and correlations were modest, although some statistically significant (paper I: Table 5). The platelet function test with the strongest correlation with the reference test was the VerifyNow® Aspirin test.

Test (units) (agonist)	Median and range (baseline) Healthy individuals	Median and range (on aspirin) Healthy individuals	Median and range (on aspirin) CAD patients	Effect size fold: median (min-max)	CV <sub>duplicate measurements (baseline, %)</sub> Healthy individuals	CV <sub>duplicate measurements (on aspirin, %)</sub> healthy / patients	CV <sub>day-to-day (on aspirin, %)</sub> healthy / patients
LTA (%) (AA 1.0 mM)	85 (76–105)	8.5 (2.5–60)	12.5 (3.5–67)	10.5	5	19 / 17	37 / 31
MEA (AU min) (AA 0.5 mM)	574 (461–976)	44 (20–79)	62 (21–219)	11.8	8	46 / 46	12 / 24
MEA (AU min) (AA 0.75 mM)	551 (434–889)	67 (24–245)	98 (12–336)	8.8	12	41 / 37	23 / 37
VerifyNow® (ARU) (AA)	662 (653–670)	425 (395–524)	433 (350–533)	1.5	0.4	3 / 3	3 / 3
PFA-100® (sec) (C-EPI)	113 (83–146)	289 (118–301)	279 (79–301)	2.2	8	16 / 27	11 / 10

**Table 1.** Coefficients of variation (CV) for duplicate measurements and day-to-day variation (duplicate measurements on 4 days) for light transmission aggregometry (LTA), multiple electrode aggregometry (MEA), VerifyNow®, and PFA-100®. Effect size was calculated for each platelet function test as the ratio between baseline and on-treatment measurements. Calculations are based on mean values of duplicate measurements for healthy individuals (n = 21) and patients (n = 43) with coronary artery disease (CAD). This table is from Grove et al [89].



**Figure 9.** Aspirin-induced platelet inhibition evaluated by platelet function tests and thromboxane metabolites. A) Light transmission aggregometry (LTA) using arachidonic acid (AA) 1.0 mM as agonist (reference test). B) Multiple electrode aggregometry (MEA) using AA as agonist at concentrations of 0.50 and 0.75 mM. C) VerifyNow<sup>®</sup> Aspirin. D) PFA-100<sup>®</sup> using the collagen-epinephrine cartridge. E) Urinary 11-dehydro-Thromboxane B<sub>2</sub>. F) Serum thromboxane B<sub>2</sub>. Open black circles indicate aspirin sensitive individuals and closed red circles indicate aspirin low-responders according to AA-induced LTA. Horizontal dotted lines indicate cut-off values for aspirin 'low-responsiveness' reported in the literature. Arrows indicate where aspirin low-responders are expected. This figure is from Grove et al [89].

Thromboxane metabolites were measured in serum and urine to obtain a pharmacologically specific measure of aspirin-induced COX-inhibition. The VerifyNow<sup>®</sup> Aspirin test was the only assay with a positive, statistically significant correlation with S-TXB<sub>2</sub> levels ( $r = 0.41$ ). Thromboxane metabolite levels, and S-TXB<sub>2</sub> levels in particular, were low and did not differ between aspirin sensitive individuals and aspirin low-responders as defined by LTA (1.0 AA). No statistically significant correlations were observed between platelet function tests and urinary thromboxane metabolites or between S-TXB<sub>2</sub> and urinary thromboxane metabolites at baseline in healthy individuals or in patients or healthy individuals during aspirin treatment. The distribution of residual platelet aggregation measured by each platelet function test and the agreement between tests are illustrated in Figure 9.

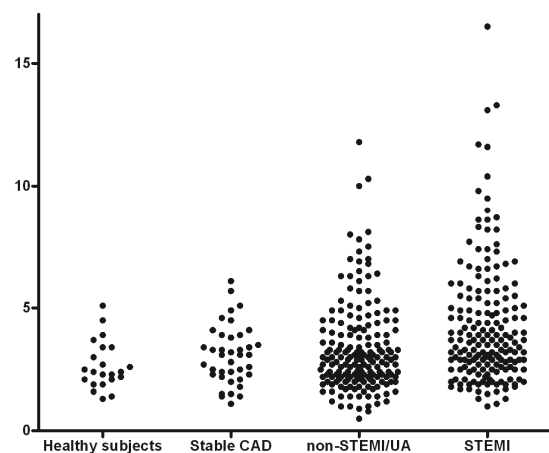
#### STUDY 2

With the primary goal of evaluating the IPF as a proxy for platelet turnover, platelet characteristics were determined in a total of 420 study participants, including healthy individuals and patients with stable CAD, unstable angina, non-STEMI, or STEMI. The main finding was an increased IPF among patients with ACS, especially in the acute phase of STEMI. Accordingly, the geometric mean [95% confidence interval] of IPF was 2.51 [2.04–3.10] in healthy individuals (group 1), 2.87 [2.45–3.36] in CAD patients (group 2), 2.93 [2.72–3.15] in the non-STEMI/unstable angina (group 3), and 3.71 [3.45–3.99] in patients with STEMI (group 4) (Figure 10). IPF differed between groups (ANOVA,  $p < 0.0001$ ), and, in particular, it was increased in patients with STEMI (group 4 vs. 3:  $p < 0.0001$ , group 4 vs. 2:  $p = 0.004$ , group 4 vs. 1:  $p = 0.001$ ).

Not surprisingly, these four groups differed with respect to a number of baseline characteristics. For example, group 1 was younger and did not include any individuals with diabetes mellitus

(paper II: Table 1). Therefore, using multivariate linear regression analysis, we tested the difference in IPF between groups for independence. In this analysis, all variables in Table 1 (paper I) that differed significantly between groups were included, and the difference in IPF remained significant ( $p = 0.0003$ ). The multivariate regression analysis also showed that the IPF seemed to be affected by smoking and diabetes mellitus. Thus, in active smokers, IPF was 18% higher than in non-smoking individuals ( $p = 0.007$ ), and in patients with diabetes mellitus, IPF was 16% higher compared with patients without diabetes ( $p = 0.060$ ).

#### Immature Platelet Fraction



**Figure 10.** Immature platelet fraction (IPF) in healthy individuals ( $n = 22$ ) and patients with stable CAD ( $n = 39$ ), non-STEMI/unstable angina ( $n = 182$ ), and STEMI ( $n = 177$ ). See text for details. This figure is from Grove et al [166].

To evaluate intra-individual variability, duplicate measurements of IPF were performed on 4 consecutive days at the same time of day for each study participant. For logistical reasons, this was not possible in patients in groups 3 and 4. In healthy individuals and patients with stable CAD, the overall-mean (i.e. mean IPF from these 4 days) and pooled standard deviation (SD) were  $2.64 \pm 0.41$  and  $3.23 \pm 0.43$ , respectively. The CV for day-to-day variation was 14% in both groups.

Since patients with unstable angina could have an underlying pathology, which is more variable (and perhaps less platelet-dependent) compared with patients suffering from MI, additional analyses were performed solely in patients with MI ( $n = 246$ ) to further explore the relation between platelet turnover and coronary thrombosis. Firstly, the difference in IPF between STEMI and non-STEMI patients remained significant ( $p = 0.025$ ) after adjusting for variables that differed significantly between groups (paper II: Table 2). Furthermore, IPF and MPV were correlated ( $p < 0.0001$ ,  $r = 0.81$ , paper II: Figure 2), and the predictive value of these parameters regarding infarct type (non-STEMI vs. STEMI) was assessed with logistic regression analysis showing a statistically significant predictive value of IPF (odds ratio = 1.35 [1.06–1.71],  $p = 0.014$ ), but not of MPV. Finally, among patients with acute chest pain (groups 3 and 4), the exact symptom duration was recorded in 208 patients, and in these patients, a weak, though significant, positive correlation between IPF and symptom duration was seen ( $r = 0.16$ ,  $p = 0.024$ ). However, when patients with a definite MI were analysed separately, this correlation did not remain significant ( $r = 0.10$ ,  $p = 0.108$ ).

### STUDY 3

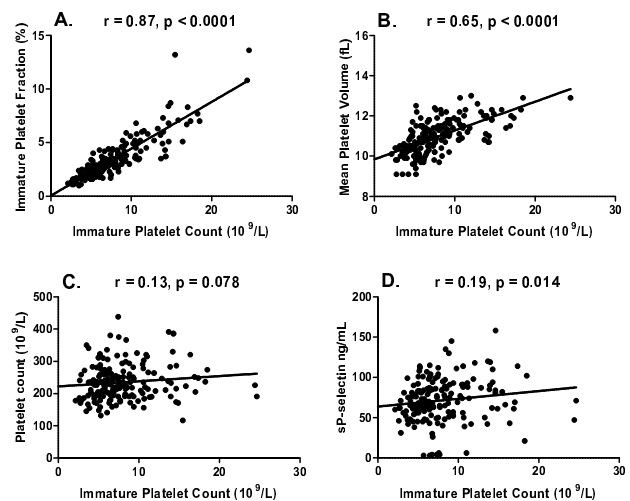
In continuation of study 2, we further explored the importance of platelet turnover. The findings in study 3 support the hypothesis that an increased platelet turnover may render aspirin less effective in patients with CAD. In particular, the data suggest that the reduced cardiovascular protection from aspirin observed in diabetics may be – at least partly – explained by an increased platelet turnover. Moreover, the study shows that commonly used platelet function tests differ in their dependence on platelet parameters such as platelet count.

To enable the investigation of the relation between platelet turnover and platelet aggregation, we included a population of patients treated with the same dose of aspirin and no other antiplatelet drugs. Contrary to patients in study 2, these patients were stable because individuals with any cardiovascular events or revascularisation procedures within the previous year were excluded. This exclusion criterion was also employed to avoid dual antiplatelet treatment with clopidogrel in addition to aspirin. Despite the exclusion of patients with recent events, the study population was not a low-risk population; it included a high prevalence of type 2 diabetics (48%) and patients with previous PCI (92%), MI (66%), coronary artery bypass grafting (23%), or stroke (10%).

Most previous studies (including studies 2 and 4) employing measurements of immature platelets to investigate platelet turnover have focused on the IPF. This study also explored other platelet parameters obtained with the XE-2100 flow cytometer as described in the above section on ‘Methods’. As shown in paper III (Table 2), no significant correlations were observed between platelet aggregation and the IPF, whereas absolute immature platelet counts (IPC) correlated with platelet aggregometry measured by MEA ( $r = 0.31$ – $0.35$ ,  $p$ -values  $< 0.0001$ ), but not with VerifyNow® results ( $r = 0.12$ ,  $p = 0.104$ ). Similarly, IPC (but not IPF)

correlated significantly with the platelet activation marker sP-selectin ( $r = 0.19$ ,  $p = 0.014$ , Figure 11).

Whole blood platelet aggregometry using MEA and VerifyNow® seemed to differ in their dependence on platelet parameters (paper III: Table 2). Thus, according to MEA, platelet aggregation correlated significantly with platelet count ( $r = 0.45$ – $0.68$ ,  $p$ -values  $< 0.0001$ ), whereas only a trend was seen with the VerifyNow® Aspirin test ( $r = 0.14$ ,  $p = 0.066$ ). Likewise, VerifyNow® results did not correlate with MPV ( $r = 0.02$ ,  $p = 0.755$ ), whereas results obtained using MEA correlated significantly with MPV ( $r = 0.17$ – $0.23$ ,  $p$ -values  $< 0.05$ ), except when aggregometry was induced by ADP. Correlations between the characteristics of platelets, white cells, and red cells are shown in Table 2. Not surprisingly, there was a negative correlation between platelet count and MPV and between platelet count and IPF. Platelet count also correlated with PDW, P-LCR, IPC (Figure 11), and with all haematological parameters. There was no correlation between platelet turnover indices and other cell lines.



**Figure 11.** Correlations between immature platelet count (IPC), immature platelet fraction (IPF), mean platelet volume (MPV), platelet count, and sP-selectin. Correlations were evaluated using Spearman’s rho. (A) IPC vs. IPF. (B) IPC vs. MPV. (C) IPC vs. platelet count. (D) IPC vs. sP-selectin. This figure is from Grove et al [167].

In order to investigate the importance of platelet parameters in patients with a low antiplatelet effect of aspirin, patients with high vs. low RPR were compared (paper III: Table 3). As no standardised definition of RPR exists, we defined RPR as the upper tertile of platelet aggregation and compared these patients with the lowest tertile of platelet aggregation. In aspirin low-responders, platelet counts (MEA:  $p < 0.0001$ , VerifyNow®:  $p = 0.055$ ) and IPC (MEA:  $p < 0.001$ , VerifyNow®:  $p = 0.060$ ) were higher, whereas IPF was not. MPV was slightly increased according to MEA induced by AA ( $p = 0.038$ ) and collagen ( $p = 0.055$ ). Furthermore, among patients with RPR according to AA-induced aggregometry, there were significantly more patients with diabetes mellitus (61% vs. 41%,  $p = 0.027$ ) and slightly higher levels of sP-selectin ( $p = 0.070$ ) and serum thromboxane  $B_2$  ( $p = 0.034$ ).

A number of small but significant differences were observed between CAD patients with and without type 2 diabetes mellitus. Although all study participants were treated with the same dose of aspirin, patients with diabetes mellitus had significantly higher

	Platelet count (10 <sup>9</sup> /L)	MPV (fL)	PDW (fL)	P-LCR	Red cell count (10 <sup>12</sup> /L)	Haemoglobin (g/dL)	Reticulocytes (%)	White cell count (10 <sup>9</sup> /L)
PC (10 <sup>9</sup> /L)	-	r = - 0.30 p < 0.0001	r = - 0.33 p < 0.0001	r = - 0.29 p < 0.001	r = - 0.19 p = 0.017	r = - 0.23 p = 0.002	r = - 0.20 p = 0.011	r = 0.31 p < 0.0001
MPV (fL)	r = - 0.30 p < 0.0001	-	r = 0.95 p < 0.0001	r = 0.99 p < 0.0001	r = 0.10 p = 0.210	r = 0.15 p = 0.051	r = - 0.06 p = 0.431	r = - 0.05 p = 0.523
IPF (%)	r = - 0.32 p < 0.0001	r = 0.78 p < 0.0001	r = 0.79 p < 0.0001	r = 0.76 p < 0.0001	r = 0.13 p = 0.096	r = 0.09 p = 0.231	r = 0.07 p = 0.388	r = 0.01 p = 0.926
IPC (10 <sup>9</sup> /L)	r = 0.13 p = 0.078	r = 0.65 p < 0.0001	r = 0.64 p < 0.0001	r = 0.63 p < 0.0001	r = 0.06 p = 0.451	r = - 0.01 p = 0.904	r = - 0.03 p = 0.693	r = 0.17 p = 0.022

**Table 2.** Platelet characteristics and haematological parameters in 177 patients with coronary artery disease. IPC = immature platelet count; IPF = immature platelet fraction; PDW = platelet distribution width; PC = platelet count; P-LCR = platelet large cell ratio; MPV = mean platelet volume.

levels of serum thromboxane B<sub>2</sub> (p < 0.0001) and higher levels of residual AA-induced platelet aggregation (p = 0.009). Furthermore, sP-selectin (p = 0.005) and thrombopoietin levels (p < 0.001) were significantly higher in diabetics.

Finally, using multivariate regression analysis, determinants of platelet turnover were investigated. Analyses were performed to identify determinants of the relative (IPF) and absolute (IPC) number of immature platelets. The IPF was affected by platelet count (p < 0.001), whereas this was not the case for the IPC (p = 0.219). Both IPC and IPF were significantly affected by smoking, diabetes status, and thrombopoietin levels. In these regression analyses, IPC and IPF were elevated by 17–21% in diabetics and smokers (p-values < 0.05).

#### STUDY 4

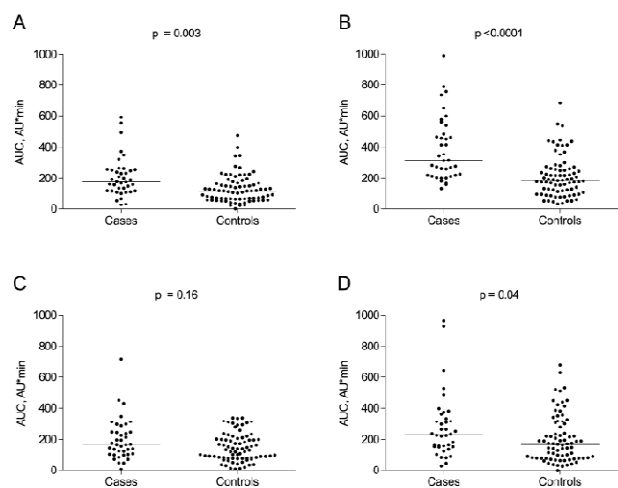
In study 4, platelet turnover and the antiplatelet effect of aspirin were evaluated in a nested case-control study in 117 patients previously undergoing PCI. Patients with angiographically verified ST were compared with matched controls with no history of ST. The main finding was a reduced antiplatelet effect of aspirin in patients with previous ST, which may be explained by an increased platelet turnover.

A detailed description of the study population and baseline characteristics is provided in paper IV (flowchart and Tables 1 and 2). The median time from index PCI to onset of ST was 10 days. Cases and controls were carefully matched at a 1:2 ratio with respect to the following risk factors for ST: age, sex, stent type, and indication for PCI. As shown in Table 1 (paper IV), the two groups differed with respect to previous MI and previous PCI, which is explained by the fact that all case patients suffered an additional MI and underwent PCI as part of their ST. Cases and controls also differed significantly with respect to other baseline variables and therefore, when analysing the antiplatelet effect of aspirin, we adjusted for these variables, together with diabetes mellitus, which has previously been reported to affect the effectiveness of antiplatelet drugs [168-171]. Analyses of IPF were adjusted for the same variables.

As shown in Figure 12, patients with previous ST had an increased RPR compared with patients without previous ST. This finding was consistent across all the types of agonists and anticoagulants used. Residual platelet aggregation was also higher in ST patients according to the VerifyNow® Aspirin test, but these results did not reach statistical significance (p = 0.12, paper 4: Figure 3). Furthermore, the platelet activation marker sP-selectin did not differ between groups.

It has been suggested that hirudin is the most suitable anti-coagulant for in vitro studies of platelet aggregation in whole blood [98], and, therefore, we recently started using this anticoagulant in addition to the most widely employed anticoagulant, citrate. In studies 1, 3, and 4, platelet function tests were performed in citrated blood (3.2% sodium citrate), and in study 4, hirudinised blood samples (hirudin 25 µg/mL) were used as well. Regardless of the agonist used, we observed a more potent inhibition of platelet aggregation in citrated blood compared with hirudinised blood (all p-values < 0.05).

Platelet turnover, evaluated using the IPF, was slightly increased among patients with previous ST, but after adjustment for the above-mentioned variables, this difference was no longer significant (p = 0.13, paper 4: Figure 4).



**Figure 12.** Platelet aggregation in 117 patients with coronary artery disease: 39 patients with previous stent thrombosis (cases) and 78 patients with no history of stent thrombosis (controls). Aggregation was induced by collagen 1.0 µg/mL in (A) citrated and (B) hirudinised blood as well as by arachidonic acid 1.0 mmol/L in (C) citrated and (D) hirudinised blood. AU = aggregation units; AUC = area under the aggregation curve; MEA = multiple electrode aggregometry (Multiplate®). This figure is from Würtz, Grove et al [172].

## DISCUSSION

Antiplatelet therapy has become a cornerstone in the prevention and treatment of CVD, with aspirin being the most widely used drug for many years. Aspirin effectively reduces morbidity and mortality from CVD, but some patients experience cardiovascular events despite taking their daily aspirin. This fact has caused a huge interest in 'resistance' to aspirin and other antiplatelet drugs. This thesis evaluates and compares a number of widely used platelet function tests and explores platelet turnover as a potential mechanism of reduced antiplatelet effect of aspirin. A comprehensive discussion of the results of studies 1–4 is provided in papers I–IV. In the following section, the phenomenon of 'aspirin resistance' and selected study results are discussed.

### THE IMPORTANCE OF OPTIMAL COMPLIANCE

Non-compliance is an obvious suspect when patients do not appear to respond to antiplatelet therapy. Thus, in a study of patients with previous MI, a total of 16% were non-compliant with aspirin, and non-compliance was associated with a 4-fold increased risk of cardiovascular events at 12 months [173]. Consistent with these findings, poorly compliant patients in the Physicians Health Study gained significantly less benefit from aspirin than compliant patients [174]. Such findings are explained by a clustering of cardiovascular risk factors in non-compliant patients [174] and of course the lack of protection from prescribed drugs not taken by these patients.

In the context of the much discussed 'aspirin resistance', the importance of optimal compliance is further stressed by the diminutive frequency of low-responders following witnessed ingestion of aspirin [79,84,175,176]. These results point to an intrinsic weakness of many previous studies that did not appropriately assess compliance, which is obviously of paramount importance when investigating the antiplatelet effect of aspirin [75,91]. In studies 1, 3, and 4, several steps were therefore taken to optimise compliance, and compliance was confirmed by measurements of S-TXB<sub>2</sub>. Owing to its high sensitivity to aspirin [49-51], S-TXB<sub>2</sub> is considered a reliable confirmation of compliance and the most specific way of pharmacologically evaluating the antiplatelet effect of aspirin [51,93,105].

### THE PHENOMENON OF 'ASPIRIN RESISTANCE'

The term 'aspirin resistance' was probably initially coined by Helgason et al [83]. Cardiovascular events in aspirin-treated patients can be explained by either non-compliance, treatment failure, or 'aspirin resistance'. Although this latter phenomenon has been intensely debated and investigated for more than a decade, it remains poorly defined and does not in any way constitute a well-defined clinical entity. Thus, no widely accepted uniform nomenclature has been established, and the clinical consequence of detecting aspirin low-responders is not clear [3,75,91]. Furthermore, the cut-off values employed in the literature for platelet function measurements to classify aspirin-treated patients dichotomically as 'responders' (sensitive) or 'low-responders' (resistant) are arbitrary and poorly validated.

Meta-analyses and reviews show that patients biochemically identified as low-responders to aspirin (often termed 'laboratory aspirin resistance') are more likely to also have 'clinical resistance' to aspirin because they exhibit significantly higher risks of recurrent cardiovascular events compared with patients who are (laboratory) aspirin sensitive [77,78,177]. Certainly, these results, with odds ratios of recurrent cardiovascular events of about 3.8 in both meta-analyses, are partly explained by insufficient control of compliance, which was only verbally assessed in the majority of

studies. Furthermore, many previous studies are hampered by the heterogeneity of study populations, a variety of different platelet function tests, and the use of arbitrary discrimination limits to identify aspirin low-responders. Still, these and other studies [178-180] show that, at least to some extent, platelet function measurements can predict cardiovascular events. However, discrimination limits to separate low-responders from patients who are sensitive to aspirin have not been successfully established.

A previous study in 223 CVD patients investigated 'aspirin resistance' using methods that directly reflect the degree of platelet COX inhibition. 'Aspirin resistance' was defined as >20% aggregation by LTA or >50% aggregation by thrombelastography. All study patients (203 undergoing PCI, 20 with previous ST) initially claimed to be aspirin compliant, and AA-induced platelet aggregation was less than 10% in all but seven PCI patients, who a posteriori all admitted being non-compliant. In these patients, in-hospital administration of aspirin decreased AA-induced platelet aggregation to less than 10%. Among the 20 patients with previous ST, one patient with 'resistance' to aspirin was identified. Thus, the prevalence of 'resistance' in the total study population was < 1% [79].

Likewise, studies using COX-specific assays and either observed aspirin ingestion [84] or platelet incubation with aspirin in vitro [82] also reported low frequencies of 'aspirin resistance', thus questioning the existence of such phenomenon. However, the fact that neither administration of large oral aspirin doses, observed aspirin ingestion nor the in vitro addition of aspirin at supra-pharmacological concentrations are fully able to eliminate the presence of (biochemical) 'aspirin resistance' shows that the phenomenon exists, although the prevalence is low [83-87]. As discussed in more detail below, this does not render the detection of low-responders to antiplatelet therapy unimportant, because identifying patients at the lower end of the Gaussian curve of platelet inhibition may result in improved therapy and reduction of cardiovascular events.

Investigation of whether largely platelet-dependent atherothrombotic events are caused by insufficient platelet inhibition by aspirin ('clinical resistance') is limited by: 1) the diagnosis of 'clinical resistance' can only be made retrospectively after a cardiovascular event and 2) the pathogenesis of atherothrombosis is multifactorial and, therefore, blocking only one of its mechanisms, platelet aggregation, cannot completely prevent atherothrombosis and its consequences [76,91]. Accordingly, it has often been argued that cardiovascular events in patients on aspirin would be better termed 'treatment failure' [181]. On the other hand, 'laboratory resistance' to aspirin has been defined as either the failure of aspirin to inhibit platelet-dependent TXA<sub>2</sub> production or failure of aspirin to inhibit platelet aggregation [76,181]. Laboratory resistance will not inevitably result in 'clinical resistance', and, vice versa; patients suffering a cardiovascular event despite aspirin treatment will not necessarily be classified as 'resistant' upon laboratory testing.

Although 'resistance', 'non-responsiveness', and 'low-responsiveness' are widely used terms, these binary definitions of platelet function likely betray its characteristic normal distribution [182]. Additionally, using 'responsiveness' has two inherent problems. Firstly, it indicates the comparison between post- and pre-treatment measurements, the latter of which are difficult to obtain in clinical cardiology. Secondly, some patients may be responders (i.e. the difference between pre- and post-treatment measurements is large) but still have post-treatment platelet aggregation levels that are very high, and, of course, the opposite

situation is also possible. Therefore, terms such as 'post-treatment platelet aggregation', 'high on-treatment platelet reactivity', 'residual platelet aggregation' and 'residual platelet reactivity' are increasingly used, as is also reflected by the change of terminology in the appended papers of this thesis. Ideally, when dichotomous definitions are used, they should be identified using receiver-operator characteristic (ROC) curve analysis to define the optimal cut-off associated with cardiovascular risk. A detailed discussion of the nomenclature is available elsewhere [76,85,181,183].

In summary, 'aspirin resistance' and similar terms have been used to describe a number of different phenomena, including the inability of aspirin to 1) prevent cardiovascular events based on the expected epidemiological effect, 2) inhibit COX-1 and platelet-dependent TXA<sub>2</sub> production, i.e. a definition based on aspirin's pharmacology, and 3) produce an anticipated effect on platelet aggregation according to in vitro measurements of platelet function, a definition based on aspirin's biological effect. These issues imply that no single assay is sufficient to cover all three phenomena.

#### MEASURING THE ANTIPLATELET EFFECT OF ASPIRIN

In its true sense, 'resistance' to any drug should be defined as the inability of the drug to inhibit its pharmacological target: true 'aspirin resistance' is only present when aspirin is not able to inhibit the COX-1 enzyme and platelet-dependent TXA<sub>2</sub> production. Accordingly, laboratory methods for evaluating the antiplatelet effect of aspirin can be categorised as COX-1 specific or COX-1 non-specific, with COX-1 specific methods primarily including thromboxane metabolites in serum or urine, but also AA-induced platelet aggregation measured by a platelet function test. In general, COX-1 non-specific methods include all platelet function assays using collagen-, ADP-, and other non-AA-agonists, but also the PFA-100® is considered among these tests. The only in vivo test available is the bleeding time test, which is now rarely used due to a considerable inter-operator variability [184]. As discussed above ('Introduction'), S-TXB<sub>2</sub>-measurements are considered the pharmacologically most specific assay to monitor aspirin's ability to inhibit COX-1-dependent thromboxane A<sub>2</sub> production [51,76,93].

#### PREVALENCE OF ASPIRIN LOW-RESPONDERS

It has repeatedly been shown that the frequency of 'resistance' is lower when COX-1-specific assays are employed and that the use of whole blood assays and point-of-care tests (especially the PFA-100®) generally results in higher frequencies of 'resistance' [79,81,130,176,185,186]. The frequency varies considerably, and a systematic review by Hovens et al. reported a mean prevalence of 'aspirin resistance' of 24% (range 0–57%) with a significantly higher prevalence in studies with aspirin dosage ≤100 mg compared with ≥300 mg [81]. Notwithstanding the fact that study 1 was not powered to thoroughly evaluate the prevalence of 'resistance', we found a low frequency of low-responders for all tests employed. Thus, among 64 study participants on aspirin 75 mg o.d. in study 1, a total of six (9%) low-responders were identified according to LTA, whereas 2 (3%) low-responders were identified when the PFA-100® was employed. No low-responders were identified according to MEA and the VerifyNow® Aspirin test. These findings are consistent with the fact that S-TXB<sub>2</sub>-levels were very low in this study as well as in studies 3 and 4. This shows that the measures taken to optimise compliance were effective, but likely also reflects the standardisation of blood sampling procedures as well as the time between aspirin ingestion and blood

sampling (1 hour). Thus, it has previously been shown that even 960 mg of aspirin is not sufficient to fully inhibit AA-induced platelet aggregation through 24 hours [50]. Moreover, a recent study found that the percentage of patients demonstrating significant recovery of AA-induced LTA and S-TXB<sub>2</sub>-measurements progressively increased during the usual 24-hour dosing interval, thus prompting the authors to conclude that 'any biological assessment of aspirin efficacy should take time since last aspirin intake into consideration' [92]. The far majority of previous studies did not standardise this time interval or they evaluated aspirin response at a later time point (2-14 hours, 14 ± 4 hours) [130,187] or at the end of the 24-hour dosing interval [79,188]. With respect to compliance it has clearly been shown that ensuring adherence is crucially important as demonstrated by the diminutive frequency of low-responders in studies employing witnessed aspirin ingestion [79,84,175,176] or in vitro addition of aspirin [82,86]. Unfortunately, as highlighted in the above-mentioned review [81], many previous studies did not report data on patient compliance.

#### REPRODUCIBILITY AND ASPIRIN SENSITIVITY OF PLATELET FUNCTION ASSAYS

In study 1 we have shown that results obtained by employing widely used platelet function tests are hampered by considerable imprecision. This finding is important to the current discussion of 'aspirin resistance', since classification of patients is usually based on single measurements with the underlying assumption that this measurement represents a stable phenotype. Previously, CVs ranging from 3 to 63% have been reported for day-to-day variation with LTA [138]. We found that CVs for duplicate measurements with AA-induced LTA during aspirin treatment were 17–19% and CVs for day-to-day variation were 31–37%, which is not impressive. The relatively high CVs for LTA are likely explained by the manual multi-step procedure. Specifically, the preparation of platelet-rich plasma might affect assay variability because platelets are very sensitive and may be activated during handling and the centrifugation procedure, which also results in the removal of large platelets. Other possible causes of low reproducibility of this assay include variations in temperature, pH, platelet count, and fibrinogen concentration [118,121,139,140]. Additionally, LTA has the drawback of being non-physiological in neglecting the assessment of interactions between platelets and other blood cells.

The CVs for MEA and the PFA-100® were moderate to high (Table 1), a finding consistent with previous studies [143,189-191]. With regard to the PFA-100®, variability of test results is likely affected by test sensitivity to many variables including e.g. the haematocrit, collagen platelet receptor density, and von Willebrand factor levels [118,141,192], although disaggregation of the blood clot inside the test device has also been suggested to explain the relatively low reproducibility [143].

Regarding MEA, results from study 3 suggest that the reproducibility may be affected by variations in platelet parameters. Thus, contrary to the VerifyNow® assay, platelet function measurements according to MEA correlated significantly with MPV, IPC, and platelet count. In accordance with these findings, a recent study in 20 healthy volunteers reported that results obtained using MEA were significantly affected by variations in platelet count below the normal range [193]. The results in study 3 extend these findings to CAD patients with normal platelet counts.

CVs for duplicate measurements at baseline, during aspirin treatment, and for day-to-day variation were all lowest for the VerifyNow® Aspirin test. A high reproducibility of this assay has also been reported in other studies [194-197], and CVs ≤3% are



indeed impressive. However, we have performed >1,800 tests in >1,100 study participants in our laboratory and have never seen ARU values below 350. As previously discussed [89], we therefore hypothesised that a fixed factor might have been added to the baseline of the ARU scale resulting in artificially low CVs (= standard deviation / mean ARU). To further explore this hypothesis, we recalculated all CVs for the VerifyNow® Aspirin test after subtracting 350 from the actual ARU values obtained. The adjusted CV for duplicate measurements at baseline was 0.9%, and the adjusted CVs for duplicate measurements during aspirin treatment in healthy individuals and CAD patients and day-to-day variation in both groups were 13–15%. Overall, although CVs for day-to-day variation with the PFA-100® were slightly lower, the VerifyNow® Aspirin assay remained the most reproducible test even after adjusting the ARU-scale.

Finally, as a measure of sensitivity for aspirin treatment, the 'effect size' was calculated for each platelet function test as the ratio between baseline and on-treatment measurements. In accordance with a previous study, MEA was most sensitive for aspirin treatment [162]. However, the effect size should be interpreted with caution, since these calculations were only based on 21 healthy individuals and, importantly, this measure is obviously dependent on the scale used by each platelet function test.

#### CONCORDANCE BETWEEN PLATELET FUNCTION TESTS AND THROMBOXANE METABOLITES

Categorising patients as normal or low-responders to antiplatelet therapy is convenient if platelet function testing is to be implemented in clinical decision algorithms and, therefore, data were both analysed as continuous and dichotomous variables. Importantly, the interpretation of correlation coefficients should be cautious as discussed below ('Strengths and limitations').

As illustrated in Figure 9, the agreement between tests was low ( $\kappa \leq 0.21$  for all comparisons), when data were analysed using cut-off levels from the literature [79,119,126-131,163-165]. Although some correlations between platelet function tests were statistically significant, the size of the coefficients were generally low (paper I: Table 5). In accordance with a recent study [198], the strongest correlation was seen between the reference method (LTA 1.0 mM AA) and the VerifyNow® Aspirin test. The fact that LTA did not correlate particularly well with any other platelet function test is probably explained by a combination of the following factors. Firstly, the imprecision (high CVs) of platelet function tests described above probably contributes to the relatively low agreement between tests. Secondly, test principles differ greatly between these assays (see 'Methods' for details).

As discussed above, thromboxane metabolite levels represent a more pharmacologically specific approach for evaluating the antiplatelet effect of aspirin. In study 1, the levels of S-TXB<sub>2</sub> and urinary thromboxane metabolites did not correlate with the reference method or any other platelet function test, except that a significant positive correlation was seen between S-TXB<sub>2</sub> and the VerifyNow® assay. The lack of correlation between thromboxane metabolites and LTA has been observed in other studies and indicates that platelet function assays cannot predict which individuals have effective inhibition of platelet-dependent thromboxane production in response to aspirin [131,199,200]. Moreover, this finding is also likely to be explained by the insufficiency of a linear model in describing the correlation between percent inhibition of S-TXB<sub>2</sub> and platelet function assays as recently reported in a study showing an initial linear and subsequent steep exponential dependence [187]. These authors also reported [187]: 1) a similar nonlinear relationship between percent inhibi-

tion of S-TXB<sub>2</sub> and urinary thromboxane metabolites thus explaining the lack of correlation between these metabolites in study 1 and 2) consistent with our findings, that no statistically significant differences in thromboxane metabolites (in both urine and serum) were observed between aspirin sensitive and low-responders according to the same widely employed discrimination limit used in study 1. These data corroborate findings in previous studies showing that, although at low doses aspirin inhibits S-TXB<sub>2</sub> formation in a dose-dependent manner, more than 95% inhibition is required to achieve full inhibition of platelet aggregation [201]. Therefore, even minimal residual capacity to generate thromboxane may be enough to sustain thromboxane-dependent platelet activation [74,187,202]. One may hypothesise that this could be particularly important in patients with an increased platelet turnover.

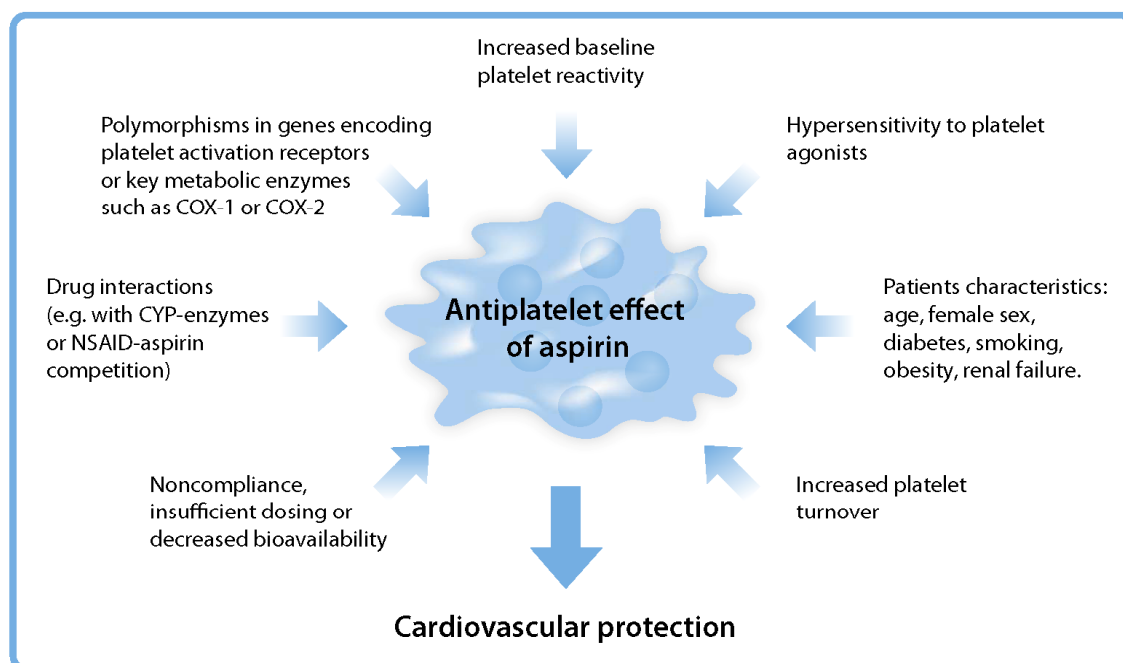
#### POTENTIAL CAUSES OF INTER-INDIVIDUAL VARIABILITY IN THE ANTIPLATELET EFFECT OF ASPIRIN

Insufficient platelet inhibition is likely explained by multiple mechanisms, many of which are not fully elucidated. Given the prevalence of atherothrombotic disease, exploring these mechanisms is important as they may provide new insight and pharmacological targets to improve the treatment of cardiovascular disease. Selected mechanisms are briefly discussed in this section, followed by separate sections on platelet turnover and diabetes mellitus. Possible causes of reduced cardiovascular protection from aspirin are summarised in Figure 13. Many of these mechanisms likely combine to cause variation in the antiplatelet effect of aspirin, with different mechanisms predominating in different patients.

Aspirin blocks only one among several pathways of platelet activation, while several other pathways remain largely unaffected (Figure 1), thus contributing to aspirin response variability. Moreover, hyper-responders to one agonist tend also to be hyper-responders to other agonists [203,204], suggesting that some individuals have increased baseline platelet reactivity and that RPR partly results from downstream signalling mechanisms such as G-proteins, phosphodiesterases etc. possibly influenced by genetic variability [205]. A study, consistent with these findings, showed that patients with a reduced antiplatelet response to aspirin also have a reduced response to clopidogrel [206].

One obvious cause of platelet response variability is reduced availability of aspirin to platelets. Reduced bioavailability might be caused by enteric-coated preparations [45,106,207,208] and, therefore, all patients in studies 1, 3, and 4 were treated with non-enteric coated aspirin only. Furthermore, reduced bioavailability may be caused by drug-interactions, the most well-documented of which is the reduced platelet inhibition observed during concomitant administration of reversible COX-1-inhibitors such as the NSAID, ibuprofen [209]. This interaction arises because of competitive inhibition by NSAIDs of the access of aspirin to the acetylation site in platelet COX-1 enzyme [209].

Other drug-interactions possibly affecting the antiplatelet effect of aspirin include COX-2 antagonists, statins, angiotensin receptor blockers, and selective serotonin reuptake inhibitors [186,210]. Furthermore, a potential interaction with proton pump inhibitors (PPIs) has been extensively discussed, especially regarding the antiplatelet effect of clopidogrel [3]. There are conflicting data concerning the effect of acid suppression with proton pump inhibitors on enteral aspirin absorption [211]. However, we have recently shown that CAD patients treated with PPIs have reduced platelet response to aspirin, as shown by increased RPR and platelet activation, compared with CAD patients not taking PPIs. The



**Figure 13.** Possible causes of reduced cardiovascular protection from aspirin. CYP: Cytochrome; NSAID: Nonsteroidal anti-inflammatory drug. Modified from Grove et al [3].

clinical implications of these findings have now been taken forward in a nationwide registry study [212].

Epidemiological data suggest that one-third of the variation in the platelet response to antiplatelet drugs is genetically determined, and several single nucleotide polymorphisms have been identified in genes involved in the biosynthetic pathway of thromboxane [213]. In particular, polymorphisms involving COX-1 and COX-2 may modify the antiplatelet effect of aspirin and perhaps predict the future risk of cardiovascular events [211,214-216]. Another example is the PIA polymorphism of the GP IIIa gene, which encodes the IIIa part of the GP IIb/IIIa receptor, the final common pathway of platelet aggregation. Although controversy exists, this polymorphism has been reported to affect the antiplatelet response to aspirin and to be more prevalent among CAD patients with MI than among CAD patients without coronary thrombosis [37,216]. Elucidation of genetic determinants of the antiplatelet effect of aspirin and other antiplatelet drugs could ultimately lead to individually tailored antiplatelet therapy based on genotyping [3,217].

As noted by many previous studies, the characteristics of patients with and without high RPR may differ on several characteristics including age, sex, body mass index, fibrinogen levels, smoking habits, circulating catecholamines, isoprostane formation, COX-2 expression, and the level of proteins associated with mechanisms such as energetic metabolism, cytoskeleton, oxidative stress, and cell survival [203,218-220]. Whether all these differences represent true risk factors for reduced antiplatelet effect of aspirin or are merely chance findings in underpowered studies remains yet unknown.

In study 3, a number of platelet parameters (MPV, IPC and platelet count) were significantly elevated among patients with RPR defined as the upper tertile of platelet aggregation according to MEA. IPC is discussed in the next section on platelet turnover and may partly explain the increased MPV, because immature

platelets are partly identified on the basis of cell size (forward light scatter). Therefore, MPV and IPC are correlated as depicted in Figure 11. The finding of an association between high RPR and platelet count is consistent with a recent study in 200 CAD patients treated with aspirin. Inadequate platelet response to aspirin was defined as residual platelet aggregation  $\geq 20\%$  per AA-induced LTA, and in a multiple logistic regression model, only platelet count remained independently predictive of an inadequate platelet response to aspirin. The authors concluded that impaired platelet responsiveness to aspirin may be partly explained by increased platelet production [221].

#### PLATELET TURNOVER

As discussed above ('Introduction'), quantification of the number and fraction of immature platelets can be used as a proxy for platelet turnover [15-18]. Normally, approximately 1011 platelets are produced every single day, with an up to tenfold increased production upon blood loss. Platelet turnover has been shown to affect the antiplatelet effect of aspirin even in healthy individuals with a presumably normal platelet turnover. Thus, in a previous study, platelet aggregation (LTA), platelet activation, and S-TXB<sub>2</sub>-levels were evaluated in 60 healthy individuals before and 24 hours after a single 325-mg dose of aspirin. Baseline and post-aspirin platelet aggregation were higher in the upper than in the lower tertile based on the percentage of immature, reticulated platelets, and among individuals in the upper tertile, the expression of P-selectin, COX-2, GP IIb/IIIa and RPR (according to ADP-induced LTA) were increased [24]. Concurrent with these findings is another study in healthy individuals showing significant recovery of COX-dependent platelet aggregation within 24 hours, even though supranormal aspirin doses of 960 mg were administered [50].

A study in 372 ACS patients on dual antiplatelet therapy with aspirin and clopidogrel, evaluated platelet turnover using the

same type of automated flow cytometer as employed in our studies [20]. The results concur with our findings in study 3. Thus, moderate but significantly positive correlations were observed between platelet aggregation, MPV, and immature platelets [20]. Similar to our findings, patients with RPR (according to LTA) had higher MPV and immature platelet parameters, than patients without RPR. In our study, the group of aspirin low-responders was also characterised by a higher frequency of patients with diabetes mellitus and higher levels of sP-selectin and S-TXB<sub>2</sub>, suggesting suboptimal aspirin-induced COX-inhibition in these patients. Our findings are in accordance with previous studies showing that almost complete COX-inhibition is required to achieve full inhibition of platelet aggregation [74,187,202]. These and other studies highlight the potential role of platelet turnover and immature platelets in the pathophysiology of ACS [20,24,222].

Study 2, the largest study until now investigating immature platelets, showed that the IPF was significantly elevated in patients with ACS, especially in patients with STEMI. This finding is in accordance with a previous smaller study [223]. Apart from the number of study participants, our study has the advantage of using an automated technique with no need for any pre-analytical steps. Previously, the determination of immature platelets was affected by a number of variables, including the type and concentration of RNA stain, incubation time, temperature, centrifugation, and resuspension, whereas analysis-related variation is likely to be minimised with the automated technique employed in our study [16].

The main findings of study 2 have recently been confirmed in a study employing the same automated flow cytometer. Thus, Gonzalez-Porras et al. investigated immature platelets in 202 patients with a documented first episode of ACS and 202 age- and sex-matched healthy individuals [224]. These authors reported that the IPF was significantly higher in ACS patients than in matched controls and, again similar to our findings, that the IPF was higher in ACS patients than in patients with non-STEMI ACS [224].

The elevated number of immature platelets observed among ACS patients in our study may partly explain previous findings of increased platelet reactivity in ACS, as indicated by reduced bleeding times and shorter closure times with the PFA-100® [225-227]. Furthermore, the differences between patients with acute and stable CAD (Figure 1) seem to concur with previous studies showing that in patients with MI, the platelet response to aspirin improved 24 to 48 hours [228] and one year [82] after the infarct. Similarly, it has been shown that patients with MI, in particular those with STEMI, have enhanced platelet reactivity [229] and a higher prevalence of RPR according to the PFA-100® than patients with non-STEMI or chest pain but no sign of cardiac disease [230]. In study 4, we evaluated platelet turnover and the antiplatelet effect of aspirin in a nested case-control study in patients with previous angiographically verified ST compared with matched controls with no history of ST. The main finding was a reduced antiplatelet effect of aspirin in patients with previous ST. Platelet turnover, as indicated by the IPF, was only slightly increased among patients with previous ST, and in adjusted analysis, this difference was not statistically significant ( $p = 0.13$ , paper 4: Figure 4). The slightly increased platelet turnover was consistent with the findings in studies 2 and 3. However, we were not able to recruit as many patients as expected and, as discussed in paper IV, we actually included fewer case patients than estimated by the sample size calculation. Still, study 4 is the largest study to specifically investigate the antiplatelet effect of aspirin in patients

with angiographically verified ST defined according to the Academic Research Consortium criteria [90].

There are several possible explanations for the findings on immature platelets and platelet turnover in studies 2–4. Firstly, residual platelet aggregation during treatment with aspirin may be affected by the haemostatic potential of immature platelets as indicated by study 3 and previous studies [20,24,26,167,231]. The RNA content of newly formed, immature platelets provides a likely explanation for the increased reactivity of this platelet subpopulation. Although the platelet mRNA is unstable and degrades within approximately 24 hours in the circulation [22,23], it enables immature platelets to synthesise proteins despite the lack of a nucleus [232]. Accordingly, the production of membrane receptors, enzymes and alpha-granule proteins have been reported, with specific examples including fibrinogen, von Willebrand factor, P-selectin, GP IIb/IIIa ( $\alpha$ IIb $\beta$ 3), COX-1, and COX-2 [24,25,54,233]. The fact that newly formed platelets express more COX-2 is likely to be particularly important because COX-2 is not blocked by low-dose aspirin [24,54,233,234].

Additionally, the accelerated platelet turnover per se may also be important. In patients with a high platelet turnover, platelets unaffected by aspirin are introduced into the blood stream possibly causing the total platelet inhibition to be insufficient, especially during the last hours of the dosing interval [50]. Normally, the irreversible COX-inhibition by aspirin compensates for the short half-life of the drug, but accelerated renewal of the drug target may be expected to shorten the duration of COX-inhibition and perhaps even dictate a different dosing interval [105]. Furthermore, while acetylated platelets can no longer synthesise TXA<sub>2</sub>, they remain sensitive to TXA<sub>2</sub> from other cellular sources such as white cells or non-inhibited platelets [92,104]. Accordingly, if the number of immature platelets is increased because of a high platelet turnover, a substantial percentage of platelets may therefore be able to synthesise TXA<sub>2</sub>, triggering aggregation of the total platelet population. This hypothesis is in accordance with previous work of FitzGerald et al. who as early as 1983 showed that upon administration of a thromboxane synthase inhibitor, both AA-induced LTA and S-TXB<sub>2</sub>-levels progressively recovered within 24 hours [235]. Similar results were obtained in a recent study after administration of aspirin [92]. Correspondingly, in vitro experiments using mixtures of aspirin-free and aspirin-treated platelets have shown that full aggregation is seen with only 2.5% of aspirin-free platelets present [201], and more recent studies concur with these results [50,92]. A very recent study thus showed that time-dependent recovery of S-TXB<sub>2</sub>-levels and AA-induced LTA after administration of aspirin was related to platelet count, inflammatory markers, current smoking and diabetes mellitus [92]. Similarly, in multivariate linear regression analyses in studies 2 and 3, we identified smoking, type 2 diabetes mellitus, and thrombopoietin levels as significant determinants of platelet turnover.

#### DIABETES MELLITUS

It is well-known that diabetes mellitus is associated with accelerated atherosclerosis, clinically resulting in premature CAD and an increased risk of cardiovascular events [236,237]. In particular, patients with type 2 diabetes have a two- to four-fold increased risk of developing CAD, and diabetics without previous MI have about the same risk of acute coronary thrombosis as nondiabetics with a previous MI [238]. Among several mechanisms contributing to the increased risk of atherothrombotic events are clustering of traditional cardiovascular risk factors (hypertension, overweight, dyslipidaemia), hyperglycaemia, impaired fibrinolysis,

endothelial and platelet dysfunction [171,188,239,240]. Importantly, patients with diabetes mellitus seem to have reduced antiplatelet effect of aspirin as indicated by laboratory [168,171,188,239,241] and randomized controlled trials [242-244]. Different mechanisms are likely to be at play, including an increased platelet turnover [171,205,245].

In studies 2 and 3, multivariate regression analyses were performed to identify determinants of platelet turnover and in both studies, diabetes mellitus was among the variables determining platelet turnover, although this did not reach statistical significance in study 2 ( $p = 0.060$ ), which only included few diabetics. Differences in platelet turnover between CAD patients with and without diabetes mellitus were modest and not statistically significant. However, an accelerated platelet turnover in diabetics would fit well with a previous study showing that CAD patients with diabetes have increased megakaryocyte ploidy and MPV [246], as well as hypersensitivity to agonists and distinct platelet volume characteristics [247,248]. Accordingly, we observed that among diabetics in study 2, there was a trend for a higher PDW, a marker of platelet anisocytosis likely reflecting accelerated platelet production in these patients [247].

The suggestion of a reduced antiplatelet effect of aspirin in patients with diabetes mellitus was raised for the first time by DiMinno et al. who showed that despite regular schedules of aspirin (four daily doses), less platelet inhibition was still achieved compared with non-diabetics [249]. The authors linked such reduced efficacy of aspirin in diabetics to a high rate of entry of new 'young' platelets into the circulation [249]. Recently, it has been reported that diabetics on antiplatelet therapy have an increased prevalence of RPR [240,250,251] that independently predicts major adverse cardiovascular events in these patients [204]. Moreover, platelet function measurements performed in type 2 diabetics, 2 and 24 hours after aspirin ingestion showed a considerable decrease in aspirin response when testing at the end of a 24-hour dosing interval [252], thus lending further support to the potential clinical relevance of an increased platelet turnover in patients with diabetes mellitus.

#### ANTICOAGULANTS FOR ANALYSES OF PLATELET FUNCTION

In order to investigate the importance of anticoagulant type on platelet aggregation, platelet function tests performed by MEA were analysed in hirudinised as well as citrated blood in study 4. We observed a significantly stronger platelet inhibition in citrated blood compared with hirudinised blood. The difference was seen for both collagen- and AA-induced platelet aggregation and is in accordance with previous studies [98,253,254]. The more pronounced platelet inhibition by aspirin in citrated blood likely reflects the importance of extracellular calcium for platelet aggregation, including possible effects on granula release and the formation of TXA<sub>2</sub> [255]. Since hirudin does not reduce the concentration of divalent cations, it provides a more physiological environment for platelet function testing than the use of sodium citrate. The use of hirudin-treated samples for platelet function testing is therefore often recommended [253,254]. The more pronounced platelet inhibition by aspirin in citrated compared to hirudin-treated blood is only of modest concern when comparing groups of patients. However, when platelet aggregation is measured and interpreted in the context of a certain scale or response levels (i.e. low-responders vs. normal responders), the type of anticoagulant may affect results and, perhaps, ultimately influence clinical decision making.

#### STRENGTHS AND LIMITATIONS

In studies 1, 3, and 4, we investigated the antiplatelet effect of aspirin. Contrary to many previous studies, we only included patients on aspirin monotherapy to avoid the influence of other antiplatelet or anticoagulant drugs. The platelet function tests employed were thoroughly evaluated in study 1, before the initiation of studies 3 and 4. Compliance was carefully optimised and confirmed by measurements of S-TXB<sub>2</sub> levels below a 10 ng/mL limit, which has previously been reported to reflect a more than 98% inhibition of platelet COX-1 activity [104]. Although all patients were on chronic aspirin treatment, there was a run-in phase of seven days. Blood sampling procedures and the time between aspirin ingestion and blood sampling were carefully standardised. Off-treatment measurements were performed in healthy individuals, but not in patients.

In study 1, the use of repeated duplicate measurements with several platelet function tests performed 8–10 times in each study participant rendered the inclusion of a large study population impossible. We did not specifically aim to evaluate the prevalence of RPR, and as the study was not powered to address the frequency of RPR, these data should be interpreted with caution. On the other hand, the many tests performed in each study participant enabled a thorough evaluation of the reproducibility of each test, and the potential influence of diurnal variations in platelet activity was eliminated by performing tests at the same time of the day for each study participant. It was not a primary aim of the study to compare healthy individuals and CAD patients, but the inclusion of matched groups (age, gender, body mass index) would have been preferable. Data were analysed as continuous and dichotomous variables, and both analyses should be cautiously interpreted. Thus, although we used the most widely employed cut-offs from the literature, these discrimination limits are poorly validated. Similarly, when interpreting the continuous variables, it should be kept in mind that different scales are employed by the assays investigated. Also, correlation coefficients may not represent the ideal way of comparing methods as shown by Bland and Altman [256]. The Bland-Altman analysis requires that both tests compared are expressed in the same units of measurement.

Study 2 is the largest published study employing fully automated flow cytometry to investigate immature platelets and platelet turnover. Baseline characteristics obviously differed between the four study groups, but the difference in IPF persisted in an adjusted analysis. Considering between-group differences in antiplatelet therapy, no platelet function tests were performed as the interpretation of such tests would have been spurious.

To our knowledge, study 3 is the largest study employing measurements of thrombopoietin and immature platelets to investigate platelet turnover in a population of aspirin-treated stable CAD patients. Yet, regulation of platelet turnover may have been even better explored by including measurements of, for example, interleukin-6. In addition to the strengths mentioned above for studies 1, 3, and 4, this study was particularly designed to investigate the antiplatelet effect of aspirin in CAD patients with type 2 diabetes mellitus. However, a better matching of CAD patients with and without diabetes would have been preferable. The performance of off-treatment measurements in diabetics would have allowed us to explore whether the higher RPR in diabetics is explained by increased baseline platelet reactivity. Although statistically significant, many of the correlations observed were weak.

To the best of our knowledge, study 4 is the largest study to specifically investigate the antiplatelet effect of aspirin in patients

with previous angiographically verified ST. Still, we included a lower number of patients than planned on the basis of our sample size calculation. In an attempt to compensate for this, we doubled the size of the control group. The matching of patients with and without previous ST was carefully performed with respect to several important risk factors for ST. Data were analysed with appropriate statistics according to the matching ratio of study groups and further adjusted for unbalanced variables (baseline and medication) that may influence platelet aggregation. Yet, potential limitations should be considered. Firstly, despite appropriate study design and data analysis, selection bias cannot be ruled out as indicated by the flow chart in Figure 1 (paper IV). Secondly, the fact that case patients experienced one extra MI and PCI procedure as part of their ST may also per se have confounded the comparison of groups. Thirdly, the stent thromboses included may have been caused by different pathophysiological mechanisms some of which were less platelet-dependent. Thus, our study population included both acute, early, late, and very late ST, and, additionally, both bare metal and drug-eluting stents were included. Finally, the retrospective nature of our study did not allow the demonstration of any causality between ST and RPR during treatment with aspirin, and, therefore, our findings should be considered hypothesis generating.

## CONCLUSIONS

This thesis evaluates and compares a number of widely used platelet function tests and explores platelet turnover as a potential mechanism of reduced antiplatelet effect of aspirin in patients with CAD. Based on the four appended papers, the main conclusions are:

Conclusions based on platelet function tests are highly dependent on the assay used: overall, reproducibility was moderate and the correlation between tests was low. CVs for duplicate measurements at baseline, during aspirin treatment and for day-to-day variability differed markedly between tests and were lowest for the VerifyNow® Aspirin test, whereas MEA (Multiplate®) was most sensitive for aspirin treatment.

Platelet turnover is increased in patients with acute coronary syndromes, especially in the acute phase of STEMI.

CAD patients with a high platelet turnover have a reduced antiplatelet effect of aspirin.

The antiplatelet effect of aspirin is reduced in CAD patients with type 2 diabetes. These patients have significantly higher levels of thrombopoietin, increased residual S-TXB<sub>2</sub> during aspirin treatment, and higher levels of the platelet activation marker sP-selectin.

Widely used platelet function tests seem to differ in their dependence on platelet count.

Patients with previous definite ST have a reduced antiplatelet effect of aspirin, which may be explained by an increased platelet turnover.

Compared with the relative fraction of immature platelets, the absolute immature platelet count (i) has a stronger correlation with platelet aggregation, (ii) is not significantly affected by the

total platelet count, (iii) significantly correlates with the platelet activation marker sP-selectin and (iv) is stronger associated with the presence of residual platelet aggregation during treatment with aspirin.

Smoking, type 2 diabetes mellitus, and thrombopoietin levels are independent determinants of platelet turnover.

## FUTURE PERSPECTIVES

Our studies have provided new insights into the antiplatelet effect of aspirin in patients with CAD. In particular, we have presented information on the performance of widely used platelet function tests and explored increased platelet turnover as a mechanism of reduced antiplatelet effect of aspirin. Recently, we have conducted a study on platelet inhibition in patients with STEMI, and new studies are planned to further explore the antiplatelet effect of aspirin in patients with diabetes mellitus and to delineate possible genetic mechanisms affecting the antiplatelet effect of aspirin.

Although the present studies have provided new information to help unmask the phenomenon of antiplatelet drug 'resistance', important questions of scientific and clinical interest remain unanswered. Firstly, future studies should investigate the mechanisms underlying residual platelet aggregation during treatment with aspirin; this knowledge may guide the development of new therapeutic approaches to improve the treatment of patients at risk of cardiovascular events. New antiplatelet drugs are currently being developed, but improved therapeutic strategies may also include steps specifically aimed at causes of reduced antiplatelet effect (Figure 13). Secondly, despite the plethora of studies linking RPR during aspirin treatment with poor prognosis, the relationship with cardiovascular events should be more firmly established. Preferably, such studies should find the best platelet function test(s) in terms of basic test performance (reproducibility, sensitivity etc.), but should also clarify which test of platelet function would best predict cardiovascular events. Thirdly, the key focus of this research area is to establish if and how changing therapy on the basis of platelet function can improve prognosis for patients at risk of cardiovascular events. Promising results in this direction have been reported in a small elegant study by Bonello et al. [8], whereas the larger and more recent trial, GRAVITAS [257], reported no benefit from moderate dose increments in low-responders to clopidogrel (data presented at the annual American Heart Association Congress 2010). Another large study presented at this congress, the ASCET trial [258], indicated that aspirin low-responders may benefit from clopidogrel, although this finding was not statistically significant. Several ongoing studies are currently evaluating the merit of individually tailored therapy on the basis of platelet function. Whether dose increments, switching to other drugs, or supplemental antiplatelet drugs is the best strategy remains to be elucidated. Also, the issue of dosing frequency should be further investigated, as once-daily dosing of aspirin may not adequately inhibit platelet aggregation in patients with an increased platelet turnover. Even if platelet function testing can improve prognosis, other issues, including cost-effectiveness, must be considered, before platelet function testing is implemented in clinical practice.

Changing the traditional 'one-size-fits-all' approach holds the promise of reducing ischaemic events without increasing bleeding risk. However, while awaiting the results of ongoing clinical trials, the proven benefit of such a strategy is limited. Therefore, chang-

ing antiplatelet therapy based on platelet function tests should so far only be performed in the setting of clinical, preferably randomized, trials aimed at identifying the optimal strategy for the individual patient.

## SUMMARY

Cardiovascular disease is the number one cause of death globally, and atherothrombosis is the underlying cause of most cardiovascular events. Several studies have shown that antiplatelet therapy, including aspirin (acetylsalicylic acid), reduces the risk of cardiovascular events and death. However, it is well-known that many patients experience cardiovascular events despite treatment with aspirin, often termed 'aspirin low-responsiveness'. This fact has caused considerable debate: does biochemical aspirin low-responsiveness have prognostic value? Can low-responders be reliably identified? And if so, should antithrombotic treatment be changed? Is the whole discussion of antiplatelet drug response merely a result of low compliance?

Compliance should be carefully optimised, before evaluating the pharmacological effect of a drug. It is well-known that cardiovascular disease is multifactorial, and, therefore, total risk reduction is not feasible. Aetiological factors to the variable platelet inhibition by aspirin seem to include genetic factors, pharmacological interactions, smoking, diabetes mellitus, and increased platelet turnover. It is a captivating thought that antiplatelet therapy may be improved by individually tailored therapy based on platelet function testing. Ongoing studies are challenging the current one-size-fits-all dosing strategy, but the preceding evaluation of platelet function assays has not been adequate.

The overall objective of this thesis was to evaluate the reproducibility of and agreement between a number of widely used platelet function tests and to explore the importance of platelet turnover for the antiplatelet effect of aspirin in patients with coronary artery disease.

In the intervention studies (studies 1,3, and 4), optimal compliance was confirmed by measurements of serum thromboxane, which is the most sensitive assay to confirm compliance with aspirin.

In study 1, platelet function tests widely used to measure the antiplatelet effect of aspirin were evaluated in healthy individuals and patients with coronary artery disease. Pharmacology-specific metabolites were measured in urine and serum to investigate the pharmacodynamic effect of aspirin and to enable the comparison with the more global tests of platelet function. Based on repeated duplicate measurements, we evaluated the reproducibility of each test. We found that reproducibility of the classical reference method was not impressive and that the newer, so-called point-of-care tests differed markedly on reproducibility. With coefficients of variation of about 3%, the VerifyNow® Aspirin test was clearly the most reproducible test – even after correction of the official scale, which begins at about 350 aspirin reaction units and, therefore, results in artificially low coefficients of variation. Among the platelet function tests investigated, Multiplate® was most sensitive for aspirin treatment.

In study 2 we performed the hitherto largest study of newly released, immature platelets as a marker of platelet turnover. The study population included healthy individuals, patients with stable coronary artery disease, and patients with acute coronary syndromes. The main finding was an increased fraction of immature platelets in patients with ST-segment myocardial infarction, indicating an increased platelet turnover. Smoking and type 2

diabetes were identified as independent determinants of platelet turnover.

In study 3 we explored the relationship between platelet turnover and the antiplatelet effect of aspirin in patients with stable coronary artery disease. The study results support the hypothesis that an increased platelet turnover reduces the antiplatelet effect of aspirin. The main findings were: 1) platelet turnover correlated with platelet aggregation measured by Multiplate® and with sP-selectin, a marker of platelet activation. 2) Patients with diabetes mellitus type 2 had reduced antiplatelet effect of aspirin compared with patients without diabetes. 3) Widely used platelet function tests differ with respect to dependence on platelet parameters, including platelet count. 4) Smoking, diabetes mellitus type 2, and thrombopoietin were identified as independent determinants of platelet turnover. 5) The relative fraction of immature platelets has been employed in most previous studies, but in stable patients the absolute immature platelet count does not seem dependent on the total platelet count, and it has a stronger correlation with both platelet activation measured by sP-selectin and with platelet aggregation during treatment with aspirin.

In study 4 we investigated platelet turnover and the antiplatelet effect of aspirin in a nested case-control study on patients with previous definite stent thrombosis. Patients with stent thrombosis were compared with patients without stent thrombosis, with whom they were matched at a 1:2 ratio with respect to risk factors for stent thrombosis: age, sex, stent type, and indication for percutaneous coronary intervention. The study showed that patients with previous stent thrombosis have reduced antiplatelet effect of aspirin and a tendency towards increased platelet turnover.

In conclusion, widely used platelet function tests markedly differ on reproducibility, and the agreement between tests is relatively poor. An increased platelet turnover as suggested by the presence of newly formed immature platelets is important for the antiplatelet effect of aspirin, and, perhaps also for the development of acute coronary thrombosis. In the future, individually tailored antiplatelet therapy may potentially improve the benefit-risk ratio of antiplatelet therapy.

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