Aspirin in Coronary Artery Disease

An Appraisal of Functions and Limitations

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THE FOUR ORIGINAL PAPERS:

1. Würtz M, Hvas AM, Kristensen SD, Grove EL. Platelet aggregation is dependent on platelet count in patients with coronary artery disease. Thromb Res 2012;129(1):56-61.

2. Würtz M, Grove EL, Wulff LN, Kaltoft AK, Tilsted HH, Jensen LO, Hvas AM, Kristensen SD. Patients with previous definite stent thrombosis have a reduced antiplatelet effect of aspirin and a larger fraction of immature platelets. JACC Cardiovasc Interv 2010;3(8):828-35.

3. Würtz M, Hvas AM, Jensen LO, Kaltoft AK, Tilsted HH, Kristensen SD, Grove EL. 24-hour antiplatelet effect of aspirin in patients with previous definite stent thrombosis. Int J Cardiol 2014;175(2):274-79.

4. Würtz M, Grove EL, Kristensen SD, Hvas AM. The antiplatelet effect of aspirin is reduced by proton pump inhibitors in patients with coronary artery disease. Heart 2010;96(5):368-71.

1. INTRODUCTION

THE PLATELET

In 1865, during his studies of human blood in the microscope, German anatomist Max Schultze (1825-1874) described for the first time in history the presence of "*…more or less numerous, irregularly formed clumps of colourless little spherules of different sizes consisting of many little spherules together*." (1;2). With this description he provided the first evidence of platelets, and his findings were soon corroborated by the Italian medical doctor Giulio Bizzozero (1846-1901) (2;3).

Today, platelets are known as anucleate, discoid blood elements with a lifespan of 7-10 days. The concentration of platelets ranges from 150 to 400 x 10^9 per liter of blood, which far exceeds what is required to sustain adequate hemostasis (4). Platelets are formed by fragmentation of the cytoplasm of polyploid bone marrow megakaryocytes, and because they lack a nucleus, for many years platelets were considered unable to synthesize protein (5). However, the finding that messenger RNA can be readily detected in resting platelets provides evidence that platelets retain a limited biosynthetic capacity to synthesize proteins de novo (6;7). Platelets are chief effectors in the formation of the initial hemostatic plug (primary hemostasis) through an elaborate response to vascular injury. Moreover, platelets are important contributors to pathological thrombus formation and vessel occlusion when activated inappropriately. As coronary vessel occlusion potentially leads to acute coronary syndrome (ACS) and death, the role of platelets in acute cardiovascular disease can hardly be overestimated. Platelets also contribute to processes extending beyond hemostasis and thrombosis such as inflammation, innate immunity, wound healing, and maintenance of vascular integrity (8).

Platelet physiology

As outlined in Figure 1 (9), platelet-dependent thrombus formation can be arbitrarily divided into phases of adhesion, activation, secretion, and aggregation.

The inner surface of blood vessels is lined by an endothelial layer constituting an antithrombotic surface. Upon endothelial rupture, subendothelial substances are exposed to the bloodstream initiating the formation of a primary hemostatic plug. Collagen, von Willebrand factor, and platelet surface glycoproteins mediate platelet adhesion to the damaged vessel wall. Subsequent platelet activation is stimulated by local agonists such as collagen, adenosine diphosphate (ADP), epinephrine, thrombin, and thromboxane (TX) A_2 as well as by physical shear stress. All platelet activation pathways ultimately converge towards the glycoprotein IIb/IIIa receptor, a heterodimeric transmembrane receptor functioning as a key mediator of thrombus formation. Therefore, glycoprotein IIb/IIIa is often referred to as the final common pathway of platelet aggregation (10). Platelet activation implies an increase in cytosolic levels of ionized calcium and a decrease in cyclic adenosine monophosphate. The altered cytosolic milieu promotes a range of reactions, including calcium-dependent assembly of the glycoprotein IIb and IIIa subunits enabling permanent linkage between activated glycoprotein IIb/IIIa complexes on adjacent platelets via fibrinogen to consolidate the formation of stable aggregates (11). Platelets lose their discoid shape to form irregular spheres by extrusion of pseudopodia. This morphological transformation imparts a substantial

increase in platelet surface area and increases the hemostatic capacity of platelets (12).

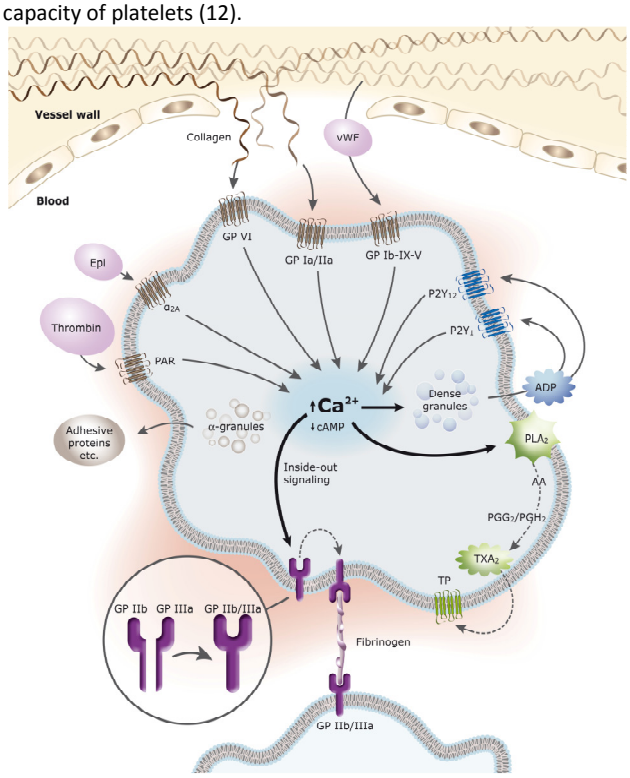

Figure 1

Platelet physiology. *Adapted from Würtz et al. (9). Platelet physiology can be arbitrarily divided into adhesion, activation, secretion, and aggregation. The circular panel shows the Ca^{2+}-dependent assembly of the GP IIb and GP IIIa subunits constituting the GP IIb/IIIa fibrinogen receptor. See main text for further details. AA = arachidonic acid, ADP = adenosine diphosphate, cAMP = cyclic adenosine monophosphate, Epi = epinephrine, GP = glycoprotein, PAR = protease activated receptor, PG = prostaglandin, PLA2 = phospholipase A_2, TP = thromboxane receptor, TX = thromboxane, vWF = von Willebrand factor.*

Platelet activation is reinforced by platelet secretion of pro-aggregatory substances from alpha-granules and dense granules (degranulation) (13). In particular, platelet release of ADP promotes platelet activation through two receptors, $P2Y_1$ and $P2Y_{12}$, of which $P2Y_{12}$ is the major receptor to amplify and sustain platelet activation in response to ADP.

Finally, increased calcium levels activate the phospholipase A_2 enzyme, which hydrolyzes platelet membrane phospholipids thereby mobilizing arachidonic acid. Arachidonic acid is converted to TXA_2; an important initiator of platelet aggregation.

The COX-1 pathway

Arachidonic acid, a 20-carbon polyunsaturated fatty acid, is the dominant precursor of eicosanoids. Eicosanoids are a group of molecules comprising prostaglandins, prostacyclins, leukotrienes, and thromboxanes. Of these, TXA_2 plays a particularly important role in platelet aggregation.

TXA_2 is produced by activated platelets and acts as a potent vasoconstrictor and stimulator of platelet aggregation mainly by increasing platelet expression of glycoprotein IIb/IIIa fibrinogen receptors. In addition, it propagates the activation signal to adjacent platelets contributing to further platelet activation and TXA_2 release, thereby initiating an amplification loop (11). TXA_2 exerts its effect during primary hemostasis and large amounts are released during platelet aggregation. TXA_2 (half-life = 30-40 seconds) is instantly hydrolyzed non-enzymatically to its biologically inert metabolite TXB_2 (half-life = 5-7 minutes), which is then rapidly metabolized to form urinary metabolites for renal clearance (14). Given the transient nature of TXA_2, measurement of serum TXB_2 or the urinary metabolites, 11-dehydro TXB_2 and 2,3-dinor TXB_2, reflects endogenous TXA_2 production more reliably than measurement of the mother compound itself (15).

Conversion of arachidonic acid to TXA_2 is catalyzed by a bifunctional enzyme complex named prostaglandin synthase. The cyclooxygenase (COX) component of prostaglandin synthase exists in two isoforms, COX-1 and COX-2, of which only COX-1 is constitutively expressed in mature platelets. COX-1-dependent TXA_2 formation occurs almost exclusively in platelets, but small amounts are released from inflammatory cells (e.g. monocytes and macrophages), from endothelial cells under physical shear stress, and from other inducible non-platelet COX-2-dependent sources (16). It follows that COX-2-dependent TXA_2 production fluctuates according to local physiological conditions. Although not accurately reflecting the endogenous production of TXA_2 *in vivo*, serum TXB_2 is a valid measure of the capacity of platelets to produce TXA_2 upon maximal stimulation. Therefore, serum TXB_2 levels reliably reflect platelet inhibition by aspirin and confirm aspirin adherence with high specificity (17;18).

Platelet production and platelet turnover

With a blood volume of 5-6 liters and a platelet lifespan of 7-10 days, an adult must produce, under non-pathological conditions, close to one trillion platelets daily to ensure that the platelet count is maintained within narrow limits. Consequently, an estimated 10% of the platelet pool is replaced every 24 hour with the renewal rate being referred to as platelet turnover. Importantly, a ten-fold increase in platelet production can be instituted under conditions of increased demand such as during surgery or inflammation (19).

The primary hormonal regulator of platelet production is thrombopoietin, a glycoprotein produced in the liver and kidneys (20). Thrombopoietin is a hematopoietic growth factor stimulating the proliferation and terminal differentiation of megakaryocyte progenitor cells making it essential for the regulation of thrombopoiesis (21). Binding of thrombopoietin to its megakaryocyte receptor stimulates the production of polyploid megakaryocytes and their subsequent differentiation into platelets. It may also directly affect platelet function by priming platelets to aggregate in response to lower levels of agonist and by rendering reversible platelet aggregation irreversible (20).

Newly formed immature platelets are large and highly reactive when released from the bone marrow. Although anucleate and free of genomic DNA, immature platelets are rich in megakaryocyte-derived messenger RNA enabling them to synthesize proteins, including COX-1, COX-2, fibrinogen, von Willebrand factor, platelet surface glycoproteins, P-selectin, and other prothrombotic substances (6;22). While blocking COX-1 effectively, low-dose aspirin inhibits COX-2 only sparsely (23). This allows immature platelets to proceed with COX-2-dependent TX generation despite aspirin treatment (24). In addition, an accelerated platelet turnover per se may increase the risk of thrombosis, since platelets uninhibited by aspirin are released from the bone marrow possibly causing the overall platelet inhibition to be insufficient (25). Platelet turnover may either be expressed as the proportion of immature platelets to the total platelet count (immature platelet fraction expressed as percentage) or as the abso-

lute number of immature platelets (immature platelet count expressed per microliter of blood).

ASPIRIN

The generic name of aspirin is acetylsalicylic acid. Low-dose aspirin displays potent antithrombotic activity, but at higher doses aspirin also holds antipyretic, analgesic, anti-inflammatory, and maybe even anti-cancer properties (26). In the context of cardiology, the therapeutic utility of aspirin spans the continuum from primary prevention through stable coronary artery disease (CAD) to ACS.

Clinical effect

A widespread appreciation of the benefit of aspirin in secondary cardiovascular prevention was founded during the 1980s. The landmark ISIS-2 trial convincingly demonstrated the superiority of aspirin over placebo when administered within 24 hours after symptom onset to patients presenting with an acute ST elevation myocardial infarction (MI) (27). At 15-month follow-up, one month of low-dose aspirin (162.5 mg, enteric-coated), either alone or in combination with fibrinolytic streptokinase, conferred a substantial relative risk reduction of nonfatal reinfarction (23%) and death (42%) (27;28). During the same period, four clinical trials documented the effect of aspirin in the setting of non-ST elevation ACS (29-32), and large meta-analyses have further cemented the clinical benefit of aspirin in patients with increased risk of cardiovascular events (33;34). A comprehensive meta-analysis encompassing more than 200,000 high-risk cardiovascular patients conclusively established that aspirin reduces by 25% the incidence of serious vascular events (non-fatal MI, non-fatal stroke, or death from a vascular cause) compared with placebo (34). These convincing data must be interpreted in light of the fact that aspirin treatment entails an increased risk, albeit statistically non-significant, of hemorrhagic stroke (35). Despite this, and the fact that hemorrhagic strokes are generally more detrimental than ischemic strokes, secondary preventive aspirin treatment displays an overall favorable risk-benefit profile also in terms of stroke (33;34). Therefore, aspirin remains mandatory in the treatment and secondary prevention of ACS and, given its low cost, is unlikely to be surpassed in any near future as a first-line remedy in cardiovascular disease.

In primary prevention, aspirin reduces the risk of a first cardiovascular event, in particular non-fatal MI (36). In this setting, however, the benefit of aspirin is offset by its propensity to cause fatal intracranial hemorrhage as well as gastrointestinal and other extracranial hemorrhages (34). Existing data thus provide sparse, if any, encouragement for the general use of aspirin in primary prevention (34), not even in high-risk populations such as diabetic patients (37).

Pharmacology

Once ingested and absorbed from the stomach and upper intestine, aspirin appears in the blood within 10 minutes to reach its peak plasma concentration after 30 to 40 minutes. Aspirin is readily metabolized by blood esterases and hepatic enzymes to produce its major metabolite, salicylate. The plasma concentration of aspirin decreases with a half-life of approximately 20 minutes (38). The inhibition of platelet function by aspirin results from blockage of COX-1. Aspirin irreversibly inhibits COX-1 by acetylating a serine moiety thereby preventing arachidonic acid from accessing the catalytic site of the enzyme (4). Since arachidonic acid is the substrate of COX-1, TXA_2 levels are hereby reduced.

Aspirin has a higher affinity for COX-1 than for COX-2 and aspirin inhibits COX-1 50 to 100 times more potently than COX-2 (16). The inhibition of COX-1 is virtually complete even at low doses (30-150 mg/day). In addition, the inhibition is rapid, dose-independent, and largely irreversible because mature platelets retain only limited capacity to re-synthesize COX-1. Therefore, considering the short plasma half-life and the permanent platelet inhibitory effects of aspirin, there is a remarkable dissociation between the pharmacokinetic and pharmacodynamic features of this drug (24).

Sufficient COX-2 inhibition requires considerably larger doses and a shorter dosing interval because COX-2 is expressed by nucleated cells capable of re-synthesizing COX-2 (23). Accordingly, aspirin must be administered in analgesic or anti-inflammatory doses (500-1,000 mg) several times daily to sustainably inhibit the COX-2 system (39).

Historically, the idea that inhibition of platelet TXA_2 formation by aspirin is non-linearly related to platelet activation has been prevailing (40;41). This would imply that virtually complete suppression of COX-1 activity was required in order for pharmacological COX-1 inhibition to translate into functional platelet inhibition. However, a recent study documented an almost perfectly linear relationship between platelet aggregation, platelet activation, and COX-1 activity (42) leaving this matter open for further investigation.

Dosing

The benefit of aspirin in the treatment of atherothrombotic disease was founded on studies performed more than 30 years ago (30;31;43). At that time, aspirin was used at higher doses than what is recommended today. Current clinical guidelines endorse the use of low-dose aspirin (75-162 mg) administered once daily in patients with CAD (44;45). This treatment strategy reflects the assumption of low-dose aspirin sustaining adequate platelet inhibition through 24 hours. However, accruing evidence questions this assumption. In fact, the dose-dependency of aspirin's antiplatelet effects has been studied for decades (38;46;47), but more recently the dosing frequency has attracted particular attention. A gradual increase in platelet function through the 24-hour dosing interval has been documented (48-53) and twice-daily dosing has been suggested (54-56).

Response variability

Acute cardiovascular events occur even in patients treated with aspirin. This is biologically plausible given the etiological heterogeneity of cardiovascular events and the modest pharmacological potency of aspirin. Although the antithrombotic properties of aspirin are widely accepted, several studies have questioned its effect in a wide span of cardiovascular patients. The rate of recurrent on-aspirin cardiovascular events remains nearly 13% during two-year follow-up indicating that not all individuals derive optimal antithrombotic effect from aspirin (33;34).

The phenomenon of reduced platelet response to aspirin may refer to 1) the failure of aspirin to inhibit platelet TXA_2 synthesis or in vitro platelet aggregation, or 2) the failure of aspirin to prevent cardiovascular events in patients prescribed aspirin (often termed "treatment failure"). So far, estimates of the prevalence of patients with reduced biochemical platelet response to aspirin reveal considerable inconsistency. Estimates vary from 5% to 65% in cardiovascular patients (57), depending on the method used to

assess aspirin response, the population being investigated, the dose of aspirin used, and the efforts made to verify aspirin adherence. Since introduced under the name "aspirin resistance" by Helgason *et al*. in 1994 (58), more than 500 publications have addressed this topic (41).

It has been heavily discussed whether a reduced biochemical response to aspirin increases the risk of cardiovascular events. Krasopoulos *et al*. reviewed 20 studies totaling 2,930 cardiovascular patients, and they identified patients with high on-aspirin platelet aggregation at an almost four-fold risk of cardiovascular events and a six-fold risk of dying (59). These findings were echoed by Snoep *et al*. (57), although both meta-analyses suffered from considerable heterogeneity among the studies included. In a more recent meta-analysis (n = 22,441), the association was much weaker, but still a doubled risk of cardiovascular events was seen in patients with high on-aspirin platelet aggregation (60).

The mechanisms underlying reduced antiplatelet effect of aspirin are complex and likely comprise clinical, pharmacodynamic, biological, and genetic elements (Figure 2) (61).

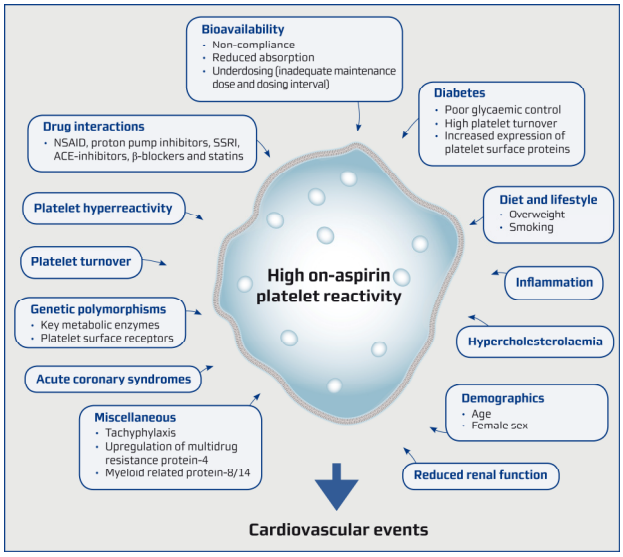

Figure 2

***Potential causes of reduced antiplatelet effect of aspirin**. Adapted from Würtz & Grove (61). ACE = angiotensin converting enzyme, NSAID = non-steroidal anti-inflammatory drug, SSRI = selective serotonin reuptake inhibitor*

PLATELET FUNCTION TESTING

The history of platelet function testing began in 1901 with the appreciation of bleeding time by Milian (62). In 1910, William W. Duke introduced the bleeding time as an *in vivo* measurement of platelet function (63), and Ivy and colleagues suggested a modified bleeding time test in 1941 (64). Bleeding time was the first test of platelet function to be used in clinical practice. This test provides the only commonly used measure of *in vivo* platelet function, given that all contemporary platelet function tests are performed in vitro. However, due to substantial operator dependency and poor reproducibility, bleeding time is no longer used routinely (65).

Light transmittance aggregometry was invented in the early 1960s (66;67) and since then it has been considered the gold standard in platelet function testing. The optical detection system is based on changes in turbidity measured as a change in light transmittance proportional to the extent of platelet aggregation. In brief, light transmittance increases as activated platelets form aggregates. Light transmittance aggregometry is performed on platelet-rich plasma, which is considered a less physiological milieu than whole blood. Moreover, the test is time-consuming, labor-intensive, and subject of considerable operator and interpreter dependency (65).

During the last decade, a range of platelet function tests have been recognized as strong alternatives to conventional light transmittance aggregometry. Most of these tests are based on whole blood and resemble physiological conditions better than tests based on platelet-rich plasma. Moreover, many newer tests share three important features: 1) they can be performed with a minimum of operator expertise, 2) they hardly depend on the operator of the instrument or the interpreter of test results, and 3) they comply with clinicians' need for rapid answers 24 hours a day (65).

It remains uncertain to what extent test results obtained with different platelet function tests are influenced by levels of whole blood compounds such as red blood cells, white blood cells, and, most importantly, platelets.

PERCUTANEOUS CORONARY INTERVENTION

Cardiovascular disease is a leading cause of death in Europe (68) and comprises a major threat to global health (69). The dominant manifestation of cardiovascular disease is atherosclerotic CAD.

Invasive management of CAD has evolved dramatically during the last 40 years, starting with the launch of coronary artery bypass surgery in the early 1960s. Almost 15 years later, another landmark was reached with the conception of percutaneous coronary intervention (PCI). Thanks to continuous improvements in stent technologies and PCI performance, PCI has become the predominant invasive treatment of CAD.

PCI is based on the inflation of a balloon within the stenotic coronary artery to press cholesterol-laden plaques into the vessel wall in order to restore free passage of blood through the coronary circulation. Generally, balloon angioplasty is followed by deployment of a stent at the site of coronary artery damage. A stent is a metallic scaffold implanted at the inflated site of the coronary artery to seal plaque rupture and to prevent elastic recoil and remodeling. During PCI, the operator advances a deflated balloon on a catheter along the arterial system to reach the stenotic coronary artery in the heart. Access to the arterial circulation is gained through the inguinal femoral artery or through the radial artery in the arm.

STENT THROMBOSIS

Coronary stenting is superior to conventional balloon angioplasty in reducing restenosis and long-term morbidity (70;71), but it entails a risk of in-stent thrombus formation referred to as stent thrombosis (ST). ST has been the major shortcoming of coronary stents since their inception, and ST mortality remains high. The interventional procedure itself unavoidably injures the vessel wall activating mechanisms such as platelet and fibrin deposition, inflammatory cell infiltration, migration and proliferation of

smooth muscle cells, and, finally, reendotheliazation (72). This leads to morphological changes of the vessel wall potentially contributing to the formation of a stent thrombus. Acute (within 24 hours) and early (between 24 hours and 30 days) ST largely result from elastic recoil and mural thrombus formation caused by periprocedural vessel injury, whereas late (between 30 days and 1 year) and very late (beyond 1 year) ST are primarily caused by intimal proliferation and chronic morphological changes as part of arterial remodeling (73).

Two different stent types are used: bare-metal stents and drug-eluting stents. Both are flexible metal tubes that turn into meshes upon expansion. A drug-eluting stent is characterized by a drug-eluting alloy that ensures the controlled release of immunosuppressive agents to the vessel wall. These agents inhibit inflammation and excessive cell growth for weeks to months. Drug-eluting stents are superior to bare-metal stents in reducing in-stent restenosis (74) and target lesion revascularization (75). They may also reduce adverse clinical events (MI, cardiac death, etc.) (76;77), but contrasting results have been published (78). Importantly, first-generation drug-eluting stents may increase the risk of late and very late ST (75-77;79;80), but new-generation stents do not seem to have this downside (80). Pathological studies suggest that drug-eluting stents, especially first-generation stents, are associated with delayed arterial healing and hypersensitivity reactions resulting in chronic inflammation, which may predispose to late and very late ST (81).

Due to the risk of ST, antiplatelet therapy is imperative following PCI. European guidelines provide a class IA recommendation for the use of aspirin and a $P2Y_{12}$ inhibitor in patients with ACS treated with PCI (82). Aspirin is recommended indefinitely, while $P2Y_{12}$ antagonists should generally be halted after 1-12 months depending on stent type and presence/absence of ACS (82). Recent and ongoing studies with new generation drug-eluting stents will clarify if short duration of $P2Y_{12}$ antagonism is favorable (83;84), acknowledging that the highest density of ST events is seen within the first month of PCI (79) and that prolonged dual antiplatelet therapy is associated with increased bleeding risk (84).

PROTON PUMP INHIBITORS

Cardiovascular protection by aspirin accrues at the expense of increased gastrointestinal bleeding risk (85). This makes aspirin the dominant contributor to gastrointestinal bleeding-related mortality (85). Moreover, gastrointestinal discomfort is an important cause of aspirin non-adherence as reflected in the pivotal CAPRIE trial. CAPRIE affirmed modest benefit of clopidogrel over aspirin in patients at risk of recurrent ischemic events, yet without leading to regulatory approval of the superiority claim (86). In CAPRIE, 40% of patients who discontinued aspirin treatment did so because of dyspepsia (86;87).

Preventive use of proton pump inhibitors (PPI) concomitant to aspirin is widely recommended (88) reflecting that gastrointestinal bleeding is a potentially life-threatening event, especially in patients with ACS (89). Yet, even in cardiovascular patients who continue aspirin treatment after suffering a gastrointestinal bleeding event, aspirin seems to confer a net clinical benefit because the risk of bleeding is outbalanced by improved cardiovascular outcome (90).

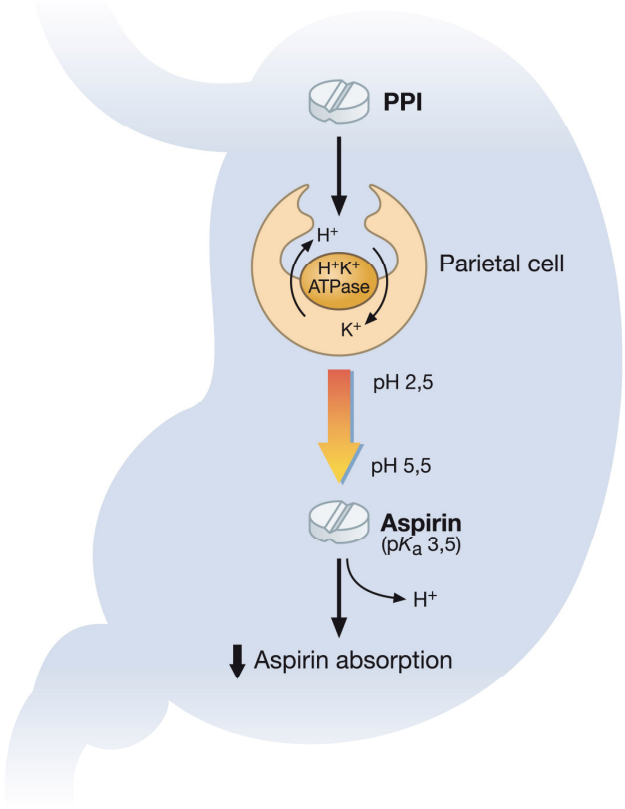

Figure 3
Suggested biochemical background for a drug interaction between aspirin and proton pump inhibitors. *Adapted from Würtz & Grove (97). Under physiological conditions, aspirin is absorbed in its non-ionized lipid state across the gastric mucosal barrier. Proton pump inhibitors inhibit the H^+/K^+-exchanging ATPase of the gastric parietal cells. Intragastric pH rises above the pK_a (3.5) of aspirin and reduces the lipophilicity of aspirin thereby lowering its gastric absorption. PPI = proton pump inhibitor.*

Since 2006, much controversy has surrounded the combined use of antiplatelet drugs and PPI (91;92). In particular, PPI have been repeatedly shown to reduce the pharmacodynamic and clinical effect of clopidogrel (93-95), although the pivotal COGENT study did not confirm any clinically meaningful interaction (96). Far less attention has been paid to the potential drug-interaction between aspirin and PPI. PPI reduce gastric acid production by inhibiting the enzyme responsible for gastric acid secretion from gastric parietal cells: the H^+/K^+-exchanging ATPase (Figure 3) (97). Modifying the intragastric milieu by raising pH potentially reduces the bioavailability of drugs, in particular those being absorbed across the gastric mucosal membrane, including aspirin (97). An interaction between aspirin and PPI thus seems biologically plausible and, given the vast number of patients taking these drugs in combination, even a modest interaction may have clinical impact.

2. AIMS & HYPOTHESES

The overall aim was to identify and describe functions and limitations of aspirin. In studies 1-4, the following hypotheses were tested:

STUDY 1

Aim

To investigate the association between platelet aggregometry results and whole blood compounds from all three major cell-lineages; platelets, red blood cells, and white blood cells.

Hypothesis

In whole blood platelet aggregometry, test results correlate with levels of whole blood compounds from all three major cell-lineages; platelets, red blood cells, and white blood cells.

STUDY 2

Aim

To explore whether patients with previous definite ST have a reduced antiplatelet effect of aspirin compared to patients with stable CAD.

Hypothesis

The antiplatelet effect of aspirin is reduced in patients with previous definite ST compared to patients with stable CAD.

STUDY 3

Aim

To investigate whether the antiplatelet effect of aspirin declines during the standard 24-hour dosing interval, especially in patients with previous definite ST. Furthermore, to explore the influence of platelet turnover on the recovery of platelet function.

Hypothesis

The antiplatelet effect of aspirin declines during the standard 24-hour dosing interval, especially in patients with previous definite ST. Recovery of platelet function occurs more rapidly in patients with an accelerated platelet turnover.

STUDY 4

Aim

To investigate whether the antiplatelet effect of aspirin is reduced in patients concomitantly treated with a PPI.

Hypothesis

The antiplatelet effect of aspirin is reduced in patients treated with aspirin and a PPI compared to patients treated with aspirin only.

3. METHODS

STUDY DESIGNS AND PARTICIPANTS

Detailed descriptions of study designs, study populations, inclusion criteria, exclusion criteria, and premises for sample size calculations are provided in the appended papers.

Study 1

Study 1 was a cohort study of 417 patients with stable CAD enrolled from November 2007 through April 2009. The study represents compiled data from three studies performed on patients with angiographically documented CAD and one or more of the following cardiovascular risk factors: previous MI, diabetes mellitus, and moderately impaired renal function (98-100). Moreover, 21 drug-naïve healthy individuals were included, who were originally enrolled in a study evaluating the agreement between different platelet function tests (101).

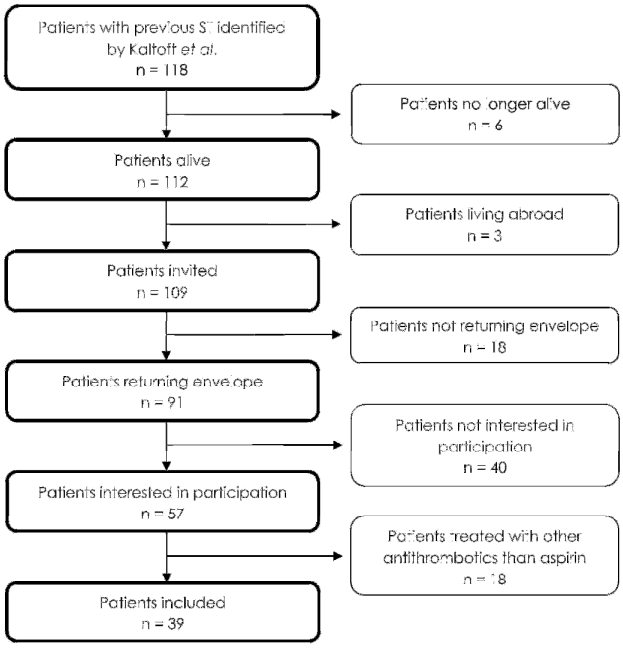

Figure 4

Flow chart of patients with previous definite stent thrombosis included in study 2. *ST = stent thrombosis.*

Study 2

Study 2 was a nested case-control study of 117 patients with CAD previously undergoing PCI. We included 39 patients previously diagnosed with definite ST and 78 control patients with no previous ST. Patients were enrolled from January through April 2009. ST patients and control patients were matched 1:2 at the individual level with respect to age, sex, stent type (bare-metal stent or drug-eluting stent), and indication for index PCI (stable angina, non-ST elevation MI/unstable angina or ST elevation MI). Patients with previous definite ST were identified according to the Academic Research Consortium criteria (102). Diagnoses of ST were adjudicated by an independent specialist committee on the basis of coronary angiographies (75). Stable CAD patients were identified in the Western Denmark Heart Registry (103). Figure 4 (98) provides an overview of ST patients included in the study.

Study 3

Study 3 was a nested case-control study of 200 participants enrolled from March through December 2012. The study cohort included 50 patients with previous definite ST, 100 patients with stable CAD (no previous MI) and 50 healthy individuals. Groups were matched 1:2:1 at the individual level with respect to age and sex. ST patients and CAD patients were further matched on diabetes. Patients with previous definite ST and stable CAD were identified as in study 2. Figure 5 (104) provides an overview of ST patients included in the study.

Study 4

Study 4 was a case-control study of 418 patients with stable CAD enrolled from November 2007 through April 2009. Among these, 364 patients were treated with aspirin and a PPI while the remaining 54 patients were treated with aspirin only. The study cohort was the same as the stable CAD cohort included in study 1. PPI use was ascertained on the day of blood sampling and confirmed by reviewing hospital records.

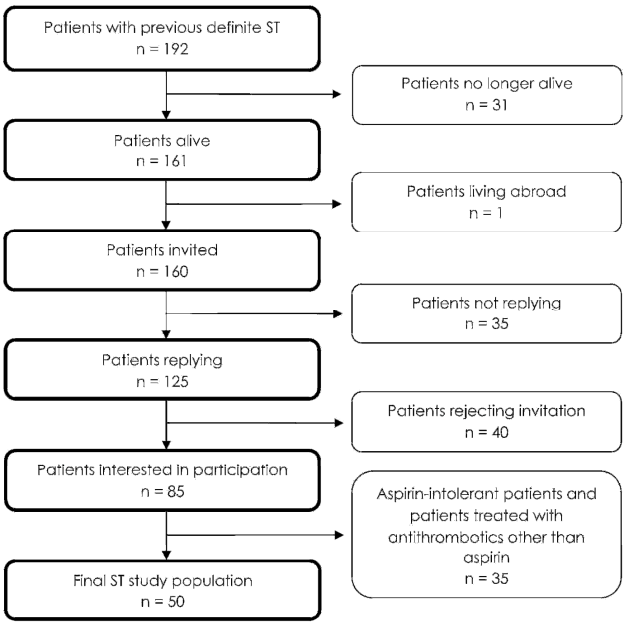

Figure 5
Flow chart of patients with previous definite stent thrombosis included in study 3. *ST = stent thrombosis.*

BLOOD SAMPLING AND ANTICOAGULANTS

In studies 1-4, blood sampling was performed exactly one hour after aspirin ingestion. In study 3, an extra set of blood samples was drawn exactly 24 hours after aspirin ingestion.

Blood was collected by venipuncture from an antecubital vein into evacuated tubes using a 19-gauge butterfly needle. Tubes were gently inverted and the first tube was discarded. Blood sampling was performed between 7:30 AM and 2:00 PM.

Blood for platelet function analysis was collected in 3.0 mL tubes containing sodium citrate 3.2% or hirudin 25 µg/mL. Blood for serum TXB_2 and soluble P-selectin analysis was collected in 5.5 mL non-anticoagulated glass tubes. Blood for thrombopoietin analysis was collected in 3.5 mL non-anticoagulated gel-tubes. Blood for analysis of hematological parameters was collected in 3.6 mL tubes anticoagulated with ethylenediamine tetraacetic acid (EDTA).

PLATELET AGGREGOMETRY

Since 2006, our research group has evaluated and compared a range of platelet function tests, including classical light transmittance aggregometry (PAP-4D aggregometer), Platelet Function Analyzer-100, Multiplate® Analyzer, and VerifyNow® Aspirin. Most recently, we tested the novel PlaCor PRT® device (105), which is based on shear stress-induced platelet aggregation and requires no stimulation with an external agonist. In these studies we identified advantages and disadvantages of each test and concluded that Multiplate® Analyzer and VerifyNow® Aspirin were superior overall (101). This is in agreement with a recent position paper issued by experts under the European Society of Cardiology (106). In studies 1-3, both tests were used, whereas in study 4 only Multiplate® Analyzer was used.

Multiplate® Analyzer

Multiplate® Analyzer (Roche Diagnostics International LDT, Rotkreuz, Switzerland) is a semi-automated whole blood platelet function test used for multiple electrode aggregometry. The system consists of a five-channel computerized device and disposable test cells (Figure 6). The test principle is based on impedance aggregometry, i.e. measurement of the increase in electrical resistance between two electrodes caused by deposition of platelet aggregates on the electrodes. Each test cell incorporates two independent sensor units enabling simultaneous duplicate analyses for internal control. Platelet aggregation is stimulated by an agonist, which is added manually using an automatic pipette. In contrast to most new tests, Multiplate® Analyzer allows for individual preparation of agonist solutions and use of different agonist concentrations. Aggregation is recorded for six minutes and platelet function is quantified as arbitrary aggregation units. Results are expressed as area under the aggregation curve (aggregation units per minute), which integrates maximal platelet aggregation (the ordinate) and aggregation velocity (the steepness of the curve).

Figure 6
Multiplate® Analyzer. *The Multiplate® Analyzer instrument. With permission from Roche Diagnostics International LTD.*

We used two different agonists to evaluate platelet function: arachidonic acid and collagen. Arachidonic acid (ASPItest; Roche Diagnostics International LDT, Rotkreuz, Switzerland) was used at a final agonist concentration of 1.0 mM and collagen (COLtest; Roche Diagnostics International LDT, Rotkreuz, Switzerland) at final agonist concentrations of 1.0 µg/mL (study 2) or 3.2 µg/mL (study 3). In study 4, only arachidonic acid was used. Agonists were stored in temperature-monitored refrigeration units and allowed to reach room temperature prior to reconstitution.

VerifyNow®

VerifyNow® (Accumetrics Inc., San Diego, CA, USA) is a platelet function test based on turbidimetric detection of platelet aggregation in whole blood (Figure 7). The VerifyNow® instrument is used with disposable cartridges containing a lyophilized preparation of fibrinogen-coated microparticles, a platelet agonist, and a buffer. The system optically detects changes in turbidity measured as an increase in light transmittance proportional to the extent of platelet aggregation. As activated platelets bind and

aggregate with fibrinogen-coated microparticles, light transmittance through the blood increases. The VerifyNow® instrument is compatible with a number of assays containing different agonists to explore specific pathways of platelet activation. We used the VerifyNow® Aspirin assay, in which platelet activation is stimulated specifically along the COX-1 pathway using arachidonic acid as the agonist. Test results are delivered within five minutes and aggregation levels are reported as Aspirin Reaction Units. The system is closed and the use of disposable cartridges containing all necessary reagents enables the system to measure platelet aggregation essentially without user interference.

Figure 7
***VerifyNow®**. The VerifyNow® instrument and a VerifyNow® cartridge. With permission from Accumetrics Inc.*

STUDY MEDICATION

All participants were treated with low-dose non-enteric coated aspirin prior to study enrolment and during study participation. The only two exceptions were the healthy individuals participating in study 1 (no aspirin) and study 3 (treated with aspirin during, but not prior to, study participation). All aspirin-treated participants were on aspirin mono antiplatelet therapy, i.e. no participant received any antiplatelet or anticoagulant drug except aspirin. Adherence to aspirin was confirmed by measurement of serum TXB_2. In order to ensure aspirin adherence and avoid pharmacokinetic heterogeneity, all participants, including healthy individuals, received a tablet box containing a one-week supply of non-enteric coated aspirin 75 mg (Hjerdyl®; Sandoz, Copenhagen, Denmark).

In studies 1, 2, and 4, patients ingested their last aspirin tablet one hour before blood sampling. In study 3, patients ingested their last aspirin tablet 24 hours before blood sampling. Immediately after blood sampling, they ingested an extra aspirin tablet (witnessed by the laboratory technician), and exactly one hour later a second set of blood samples was drawn (Figure 8).

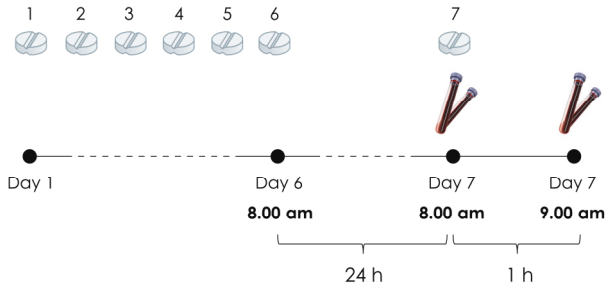

Figure 8
Time points for aspirin ingestion and blood sampling in study 3.

LABORATORY ANALYSES

Standard hematological parameters

Platelet count, red blood cells (including hemoglobin and hematocrit), and white blood cells were measured by automated flow cytometry.

Thromboxane B_2

TXB_2 is the stable metabolite of TXA_2, and TXB_2 produced *ex vivo* during whole blood clotting is an index of platelet COX-1 activity. Thereby, serum TXB_2 reflects the platelet inhibiting effect of aspirin and is a widely used marker of aspirin adherence. Measurements are mostly performed using enzyme-based immunoassays.

Serum TXB_2 was measured in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. In brief, samples were thawed and diluted with ELISA buffer to reach concentrations within the range of the standard curve. Using ELISA buffer as the matrix, serial dilution of TXB_2 between 1,000 pg/mL and 7.8 pg/mL was performed to establish a standard curve. The concentration of TXB_2 in the samples was calculated by performing a logistic four-parameter fit of the standard concentrations versus the ratio of the absorbance of a particular sample to that of the maximum binding sample. If results were outside the 20-80% range of the standard curve, samples were re-assayed with appropriate dilutions. Blood was allowed to clot for one hour at 37°C to induce maximal platelet activation and TXB_2 production. Samples were centrifuged (2,600 x g, 15 minutes) and the supernatant serum was stored at -80°C until assayed (107).

Immature platelets and mean platelet volume

The absolute and relative numbers of immature platelets (immature platelet count and immature platelet fraction) as well as the mean platelet volume were used as markers of platelet turnover. Newly formed platelets contain small amounts of rough endoplasmatic reticulum, i.e. they are "reticulated". Reticulated platelets can be stained using nucleic acid-specific fluorescent dyes allowing the differentiation between reticulated (immature) and non-reticulated (mature) platelets. This method has been increasingly used since automated flow cytometric techniques became widely available.

Complete blood counts, including immature platelet fraction and mean platelet volume, were assessed using Sysmex® XE-2100 (studies 1, 2 and 4) or XE-5000 (study 3) hematology analyzers (Sysmex, Kobe, Japan) with upgraded software (XE IPF Master) to discriminate between mature and immature platelets. Afterwards, the immature platelet count was calculated as the product of the immature platelet fraction (%) and the total platelet count (10^9/L) divided by 100 and is expressed per microliter of blood. EDTA blood was stored at room temperature until analyzed. Importantly, the use of EDTA anticoagulation might limit the validity of platelet parameters inferred from platelet size measurements because platelet size increases over time under EDTA preservation (108).

P-selectin

Selectins are a group of receptors present on platelets, endothelial cells, and lymphocytes. P-selectin (CD62P) is a cell adhesion

molecule stored in the alpha-granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon platelet activation, platelets bind to leukocytes in circulating blood to form multicellular aggregates. During this process, the interaction between platelet-bound P-selectin and the P-selectin ligand (PSGL-1) on the leukocyte is a dominant molecular event tethering platelets to the endothelium (109). Shortly after, P-selectin is shed by cleavage and released into the circulation as soluble P-selectin. P-selectin is considered resistant to *ex vivo* platelet activation (e.g. during anticoagulation or plasma preparation) making soluble P-selectin a suitable marker of *in vivo* platelet activation (110;111).

Soluble P-selectin was measured in serum as an index of platelet activation. Non-anticoagulated whole blood was allowed to clot for 30 minutes at room temperature before serum was separated by centrifugation (1,000 x *g*, 15 minutes) and stored at -80°C until assayed. The serum concentration of soluble P-selectin was determined using a commercially available ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

Thrombopoietin

Thrombopoietin was measured in serum as a marker of the activity of the thrombopoietic system. Sampling and preparation of blood was performed as described for soluble P-selectin. The serum concentration of thrombopoietin was determined using a commercially available ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

STATISTICS

All statistical analyses were performed using the Windows-based software packages STATA 12.1 (StataCorp LP, TX, USA) and GraphPad Prism 5 or 6 (GraphPad Software Inc., La Jolla, CA, USA). Graphics were performed using GraphPad Prism 5 or 6. All tests of significance were two-tailed with a probability value of p<0.05 considered statistically significant. The statistical methods used in each study are detailed in the appended papers.

Upon inclusion, each participant was assigned a unique identification number and a case report form, in which clinical characteristics, comedication, and platelet function test results were registered. To minimize typing errors, data were entered twice into a computer using the Windows-based software package EpiData Entry 3.1 (EpiData Entry, EpiData Association, Odense, Denmark) (112).

4. RESULTS

An overview of the main results of studies 1-4 is provided below, while more granular data are provided in the appended papers.

STUDY 1

The primary finding was a significant positive correlation between platelet count and platelet aggregation in aspirin-treated patients with stable CAD (n = 417) (Figure 9). Generally, correlations were only weak to moderate, and they were stronger when platelet aggregation was measured with Multiplate® Analyzer (r-values 0.36 and 0.39, p-values <0.0001) than with VerifyNow® Aspirin (r = 0.11 p = 0.03).

In multivariable analyses (adjusting for age, sex, body mass index, diabetes, smoking, red blood cells, and white blood cells), platelet count was an independent predictor of aggregation levels by Multiplate® Analyzer (both agonists), but not by VerifyNow® Aspirin.

Red blood cell levels (hemoglobin and hematocrit) correlated inversely with aggregation levels measured by Multiplate® Analyzer (collagen-induced) and VerifyNow® Aspirin, but correlations were weak (r-values -0.09 to -0.21, p-values 0.07 to <0.0001) and not consistent when using arachidonic acid with Multiplate® Analyzer (r-values 0.00 and -0.01, p-values >0.87).

White blood cell levels correlated positively with aggregation levels measured by Multiplate® Analyzer (r-values 0.25 and 0.45, p-values <0.001) and VerifyNow® Aspirin (r = 0.15, p = 0.05). Of note, white blood cells were measured only in 177 of 417 patients.

Results were robust when restricted to aspirin-naïve healthy individuals, but the precision of our correlation estimates was limited by the modest cohort size (n = 21).

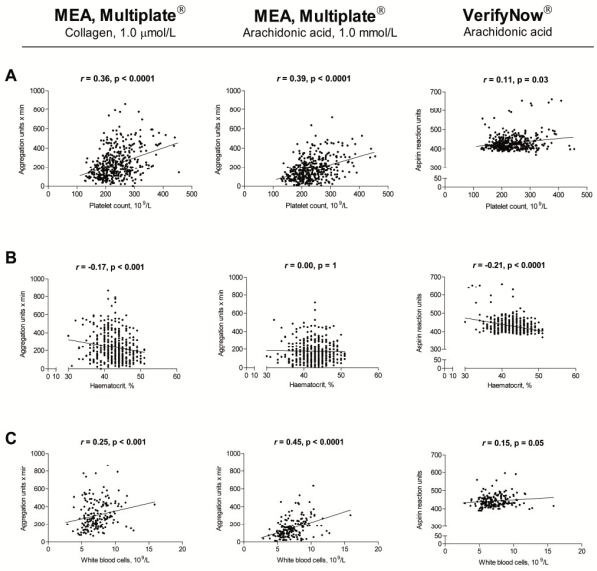

Figure 9

Correlations between platelet aggregation and whole blood parameters in 417 aspirin-treated patients with coronary artery disease (study 1). *Correlations between whole blood platelet aggregation and (A) platelet count, (B) hematocrit, and (C) white blood cells. Data on hemoglobin not shown. MEA = multiple electrode aggregometry.*

STUDY 2

The primary finding was increased platelet aggregation in patients with previous definite ST compared to patients with stable CAD. Using Multiplate® Analyzer, the difference was consistent across agonists (arachidonic acid or collagen) and anticoagulants (citrate or hirudin) (p-values 0.16 to <0.0001, Figure 10). Using VerifyNow® Aspirin, a similar trend was found, although the difference was not statistically significant (median 416 [interquartile range 404 to 434] vs. 409 [400 to 422] Aspirin Reaction Units, p = 0.12). Results were adjusted for the matched design using robust standard errors and further adjusted for smoking, PPI use, previous coronary artery bypass grafting, and diabetes.

Platelet turnover assessed by immature platelet fraction was non-significantly increased in ST patients (median 2.7 [interquartile range 2.2 to 3.8] vs. 2.3 [1.7 to 3.1] %, p = 0.13) while platelet activation assessed by soluble P-selectin was not (mean 81.0 ± 29.8 vs. 82.3 ± 22.4 ng/mL, p = 0.56).

All patients were adherent to aspirin as confirmed by suppressed levels of TXB_2 reflecting near-maximal inhibition of COX-1

activity (geometric mean 1.53 [95% confidence interval 0.67 to 2.86] ng/mL, range 0.14 to 18.18 ng/mL).

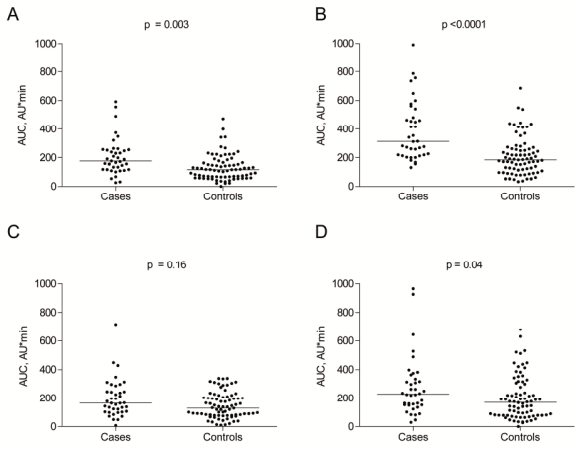

Figure 10

Platelet aggregation by Multiplate® Analyzer in 39 patients with previous definite stent thrombosis and 78 CAD patients without previous stent thrombosis (study 2). *Aggregation was induced by collagen 1.0 µg/mL in (A) citrated and (B) hirudinized blood as well as by arachidonic acid 1.0 mM in (C) citrated and (D) hirudinized blood. Comparisons made using the Mann-Whitney U test. Horizontal lines indicate medians. AU = aggregation units, AUC = area under the aggregation curve.*

A total of 26 ST patients were on aspirin and clopidogrel when suffering ST, while the remaining 13 patients were on aspirin only. All patients confirmed that they were adherent to aspirin when ST occurred. Median time from index PCI to ST was 10 days (range 0 to 1,030 days). Median time from ST to blood sampling was 1,733 days (4 years, 9 months).

The two groups were successfully matched with respect to age, sex, stent type, and indication for PCI. There was an excess of previous PCI, previous coronary artery bypass grafting, and current PPI use in patients with previous ST, which is why these variables were entered into the multivariable regression model. Of 112 living patients with previous definite ST, we were able to include only 39 patients due to the reasons outlined in Figure 4. In particular, 18 patients otherwise eligible for study participation were not included due to ongoing treatment with clopidogrel or other antithrombotic drugs.

STUDY 3

The primary finding was increased platelet aggregation 24 hours after aspirin intake compared to 1 hour after aspirin intake irrespective of the agonist used (p-values <0.0001, Figure 11). Results were consistent across study groups, except in healthy individuals when collagen was used as agonist (p = 0.4).

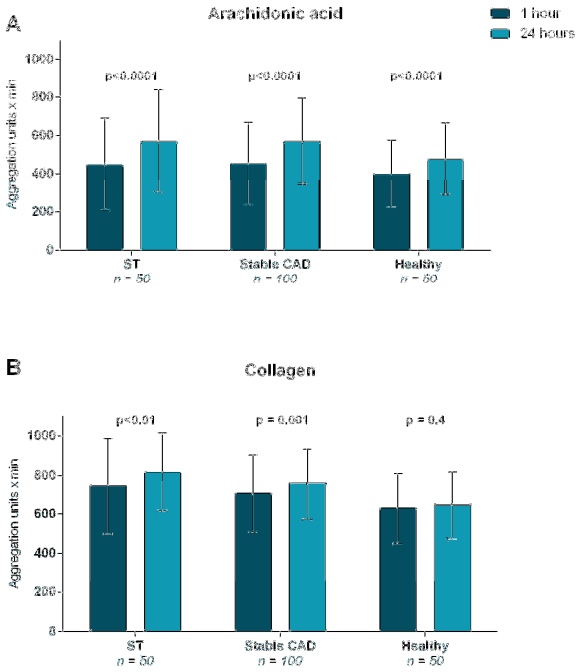

Figure 11

Platelet aggregation 1 and 24 hours after aspirin intake (study 3). *Platelet aggregation induced by arachidonic acid 3.2 mM (A) and collagen 1.0 µg/mL (B). Comparisons made using a paired t test with p-values referring to the differences in platelet aggregation between 1h and 24h samples. Boxes and whiskers represent mean and standard deviation. CAD = coronary artery disease, ST = stent thrombosis.*

The increase in platelet aggregation from 1 to 24 hours after aspirin intake did not differ significantly between groups, although ST patients displayed a higher numerical increase in platelet aggregation (arachidonic acid: p = 0.26; collagen: p = 0.16).

TXB_2 levels (p<0.0001) and soluble P-selectin levels (p<0.0001) also increased significantly from 1 to 24 hours after aspirin intake indicating increased COX-1 activity and platelet activation at the end of the dosing interval (Figure 12). No significant differences between groups were found.

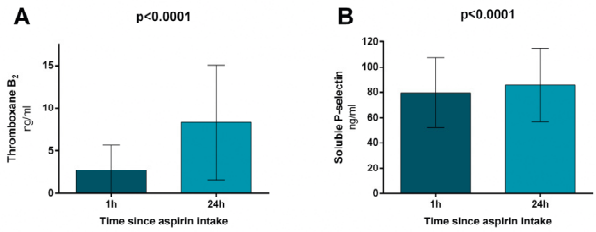

Figure 12

COX-1 activity and platelet activation 1 and 24 hours after aspirin intake (study 3). *COX-1 inhibition assessed by serum thromboxane B_2 (A) and platelet activation assessed by soluble P-selectin (B) (n = 200). Comparisons made using a paired t test. Boxes and whiskers represent mean and standard deviation. Since no inter-group differences were found, all 200 participants are pooled in this figure.*

Patients with previous ST had the highest 1- and 24-hour levels of immature platelet fraction (p-values 0.002 and 0.005), immature platelet count (p-values 0.008 and 0.003), and mean platelet volume (p-values 0.004 and 0.002) indicating increased platelet turnover compared with stable CAD patients and healthy individuals. Furthermore, they had the highest 1-hour levels of thrombopoietin (p<0.0001), whereas 24-hour levels did not differ.

All participants were adherent to aspirin as confirmed by suppressed 1-hour levels of TXB_2 reflecting near-maximal inhibition of COX-1 activity (geometric mean 1.77 [95% confidence interval 1.54 to 2.04] ng/mL, range 0.007 to 17.89 ng/mL).

Patients with previous ST and patients with stable CAD were successfully matched with respect to age, sex, and diabetes, but there was an excess of previous MI in the ST group explained by the intended selection of stable CAD patients with no previous MI. The inclusion of patients with previous ST was performed according to Figure 5.

STUDY 4

The primary finding was increased platelet aggregation in patients treated with aspirin and a PPI compared to patients treated with aspirin only (p = 0.003, Figure 13). The difference was sustained after adjustment for the following cardiovascular risk factors: age, sex, body mass index, smoking, comedication, previous MI, and diabetes (p = 0.013).

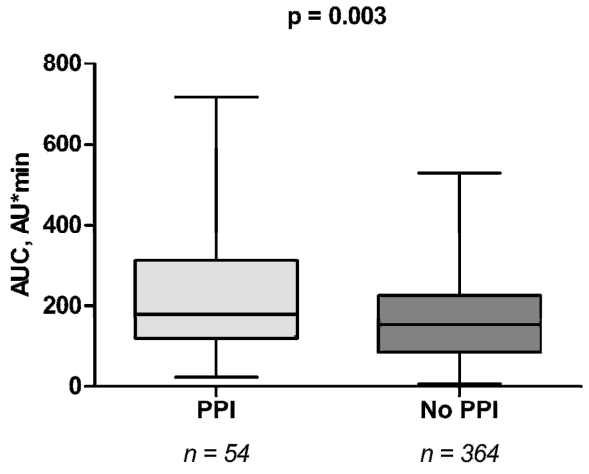

Figure 13

Platelet aggregation in patients treated with aspirin and a proton pump inhibitor and in patients treated with aspirin only (study 4)*. Platelet aggregation assessed with Multiplate® Analyzer. Horizontal lines and boxes indicate median with interquartile range. Whiskers indicate range. AU = aggregation unit, AUC = area under the aggregation curve, PPI = proton pump inhibitor.*

Likewise, platelet activation assessed by soluble P-selectin was higher in patients treated with aspirin and a PPI (median 88.5 [interquartile range 65.2 to 105.8] vs. 75.4 [60.0 to 91.5] ng/mL, p = 0.005). The difference remained significant after adjustment for cardiovascular risk factors (p = 0.013).

All patients were adherent to aspirin as confirmed by suppressed levels of TXB_2 reflecting near-maximal inhibition of COX-1 activity (geometric mean 0.96 [95% confidence interval 0.88 to 1.05], range 0.04 to 18.18] ng/mL). The two groups did not differ with respect to demographic characteristics or cardiovascular risk factors.

5. DISCUSSION

ADHERENCE TO ASPIRIN: WHY BOTHER?

Many patients do not take their medication as prescribed. Important reasons are forgetfulness, lack of information, and decision to omit drug intake (113). Essentially, only two approaches allow for valid evaluation of aspirin adherence: 1) observed ingestion of the drug or 2) measurement of the level of acetylsalicylic acid or TX metabolites in blood (114). The importance of confirming adherence can hardly be overestimated, as non-adherence has severe prognostic implications (115;116) and is the primary cause of laboratory-defined "aspirin resistance" (117;118). Nonetheless, in many previous studies proper verification of aspirin adherence has not been performed, which introduces a major risk of overestimating the true number of patients with reduced antiplatelet effect of aspirin.

ADHERENCE TO ASPIRIN: HOW TO MEASURE?

We measured TXB_2 levels in serum to verify aspirin adherence, but measurement of other metabolites may be used (119).

Being excreted renally, TXB_2 is detectable in urine. Once cleared through the kidneys, TXB_2 forms a number of metabolites, the most abundant of which is 11-dehydro TXB_2 (120). However, there are important differences between serum and urine measurements. TXA_2 formation occurs largely in platelets, but smaller amounts are released from inflammatory cells such as monocytes and macrophages, from endothelial cells under physical shear stress, and from other non-platelet COX-2-dependent sources (5). Consequently, COX-2-dependent TXA_2 production fluctuates according to local physiological conditions and serum TXB_2 does not accurately reflect the endogenous production of TXA_2 *in vivo*. Nonetheless, it serves as a valid estimate of the capacity of platelets to produce TXA_2 upon maximal stimulation and is considered the most pharmacologically specific test of aspirin's effect on platelets (17;121).

In contrast, 11-dehydro TXB_2 may be considered a suitable marker of *in vivo* TX generation as it is not formed in the kidneys. This urinary metabolite therefore represents a time-integrated index of TXA_2 biosynthesis *in vivo* (122). Urinary metabolites have a long circulating half-life and remain stable in urine, which makes them a valuable and feasible measure of TXA_2 production. Moreover, urine-based methods exclude artifacts caused by platelet activation during blood sampling. On the other hand, up to 30% of urinary metabolites derive from non-platelet sources because nucleated cells (e.g. monocytes and vascular endothelial cells) are capable of regenerating COX enzyme within the 24-hour aspirin dosing interval (123). This allows for TXA_2 production either directly or indirectly by providing prostaglandin H2 as a substrate for the platelet TX synthase in aspirin-inhibited platelets thereby bypassing COX-1 (123). Therefore, although low-dose aspirin provides irreversible and virtually complete inhibition of COX-1, urinary metabolites are not highly specific for aspirin-induced inhibition of platelet COX-1.

An even more specific way of evaluating aspirin adherence is to measure plasma levels of acetylsalicylic acid or salicylic acid. Acetylsalicylic acid is unsuitable because of its short plasma half-life, whereas salicylic acid is relatively stable with a plasma half-life of three hours. Very recently, we validated a new method to measure acetylsalicylic acid and salicylic acid concentrations by ultra high performance liquid chromatography (119). Being less time-consuming, less expensive, and more sensitive than serum TXB_2 measurement, this technique may serve as an alternative means to verify adherence to aspirin.

MONO VERSUS DUAL ANTIPLATELET THERAPY

Biochemical mechanisms explaining the antiplatelet effect of aspirin cannot be optimally characterized in studies including patients on dual antiplatelet therapy (aspirin and a $P2Y_{12}$ inhibitor) because several interdependent platelet activation pathways are simultaneously inhibited (124). Therefore, our study popula-

tions were restricted to patients treated with aspirin only, whereas many previous studies included patients treated with both drugs (125-129).

Patients with reduced antiplatelet effect of aspirin are likely to also have reduced effect of $P2Y_{12}$ inhibitors (129). This may have various explanations. Firstly, a mechanistic interdependence of the different pathways involved in platelet aggregation is plausible (130). Accordingly, TXA_2 generation is mediated in part through $P2Y_{12}$ (131;132) and, conversely, $P2Y_{12}$ inhibitors are likely to synergistically affect aspirin-induced inhibition of arachidonic acid-induced platelet aggregation (133;134). Secondly, intrinsic platelet reactivity (i.e. high platelet reactivity in drug-naïve individuals) likely contributes to a generalized reduction in the effect of antiplatelet drugs (135;136). Thirdly, at high levels of platelet turnover, large amounts of immature platelets are released to the blood, and immature platelets are not necessarily inhibited by aspirin or $P2Y_{12}$ inhibitors because of the short plasma half-life of these agents (99;137).

Studies 1-4 included only patients on non-enteric coated aspirin mono antiplatelet therapy, i.e. no other antithrombotic drugs were allowed. For two reasons, we used only non-enteric coated aspirin. Firstly, we sought to uniform pharmacokinetics across study participants. Secondly, the bioavailability of non-enteric coated aspirin is 40 to 50% (38), which is substantially higher than the bioavailability of enteric coated and sustained-release preparations (138).

CHOICE OF ANTICOAGULANT

Platelet aggregometry inherently requires *ex vivo* anticoagulation, which inhibits platelet aggregability. Various different agents possess anticoagulant properties, including citrate, lithium heparin, EDTA, lepirudin, and hirudin. For the reasons given below, we used citrate and hirudin to anticoagulate blood obtained for platelet function analyses.

Citrate remains the most commonly used anticoagulant for coagulation analyses in general. In the setting of in vitro platelet function testing, citrate prevents calcium-dependent thrombin generation and might artefactually amplify platelet inhibition exerted by antiplatelet drugs (139). We primarily anticoagulated whole blood samples with hirudin, a direct thrombin inhibitor, because it resembles physiological conditions more than citrate and preserves platelet function better (140). Hirudin blocks thrombin, which catalyzes the conversion of fibrinogen to fibrin. Subsequently, it catalyzes the activation of coagulation factor XIII, which cross-links fibrin ensuring solid clot formation. Unlike citrate, hirudin does not seem to affect levels of Ca2+ and Mg2+ (141). Generally, results obtained with hirudin and citrate correlate well, but platelet aggregation is more potently inhibited under citrate preservation, as shown in study 2.

As recommended by the manufacturer, we used citrate as anticoagulant with VerifyNow® Aspirin (142). A recent study suggests that hirudin may be more favorable, because it obviates pre-testing incubation allowing for immediate tests of platelet function (143). This is likely the case for Multiplate® Analyzer as well, as indicated in a recent study from our research group (107). Therefore, hirudin seems as the better anticoagulant used with Multiplate® Analyzer and VerifyNow® Aspirin, but since hirudin tubes are still relatively expensive, the continued use of citrate tubes is considered reasonable.

CHOICE OF AGONIST

We used two agonists, arachidonic acid and collagen, to cover different aspects of platelet function. Other agonists, including ADP, thrombin, and epinephrine, may also be used to induce platelet aggregation, but arachidonic acid and collagen were chosen for the reasons given below.

Platelet activation occurs from various stimuli, including TXA_2 and collagen, both of which converge towards the glycoprotein IIb/IIIa receptor (Figure 1). However, there are important differences between the two pathways. Firstly, they exert their effect at different stages in the cascade of reactions ultimately leading to platelet aggregation. Collagen is a direct initiator of platelet aggregation (144), whereas TXA_2 provides secondary positive feedback to reinforce platelet activation (145). Secondly, arachidonic acid-induced platelet aggregation reflects the COX-1-specific effects of aspirin, while collagen activates platelets along pathways partially bypassing COX-1 (Figure 1). Accordingly, arachidonic acid-induced platelet aggregometry is the most specific, and thus the recommended, functional test of the antiplatelet effect of aspirin (106). In contrast, collagen is less specific for the antiplatelet effect of aspirin, but it may better reflect the fact that platelets function through complex mechanisms involving multiple receptors and pathways (130).

In studies 2 and 3, aggregation results were very uniform whether based on platelet stimulation with arachidonic acid or collagen. This suggests that the differences in platelet aggregation observed in study 2 (previous ST versus stable CAD) and study 3 (1 hour versus 24 hours) were not driven by differences in COX-1 activity alone. Aspirin does indeed seem to have antiplatelet effects independent of COX-1 (146;147), which is an important consideration implying that selected stimulation of the COX-1-dependent platelet activation pathway seems insufficient as a tool to identify a reduced platelet response to aspirin. Regrettably, we used no specific off-target control (e.g. ADP or thrombin) to verify COX-1-independent effects of aspirin. It follows that the results of our aggregation analyses may indicate, but do not unambiguously confirm, COX-1-independent effects of aspirin.

CLINICAL UTILITY OF PLATELET FUNCTION TESTING

From a clinical cardiologist's perspective, the increasing interest in platelet function testing can be ascribed to the overwhelming evidence of arterial thrombosis being the predominant cause of adverse events following PCI. Recent focus has centered primarily on the ability of platelet function tests to evaluate and improve the effect of antiplatelet therapy. However, ideally a platelet function test should be able to 1) detect platelet hyperreactivity enabling secondary and perhaps even primary prevention, 2) detect intra-individual variation in platelet response to antiplatelet agents and assess the risk of recurrent arterial thrombosis forming the basis of individualized antiplatelet therapy, and 3) assess the risk of bleeding (148). No currently available platelet function test meets these demands.

To biochemically define a true aspirin responder one must compare a pre-treatment measurement with a post-treatment measurement, the first of which is rarely feasible in clinical settings. Another concern is that a patient responding strongly to aspirin (defined as a pronounced pre- versus post-aspirin drop in platelet aggregation) may still have a high level of on-aspirin platelet aggregation. This patient may be at increased thrombotic risk despite responding strongly to aspirin. Therefore, in most studies the concept of "high on-treatment platelet reactivity" is used to quantify the platelet response to aspirin. This is reason-

able given that high on-treatment platelet reactivity is intuitively more likely to be associated with adverse clinical outcomes than the pre- versus post-aspirin drop in platelet aggregation.

In the absence of a consensual standard in platelet function testing, we performed whole blood platelet aggregometry using Multiplate® Analyzer and VerifyNow® Aspirin. A recent study from our group evaluated and compared these tests (101), both of which have been used in numerous pharmacodynamic and clinical studies (65). Unlike many previous studies, however, we analyzed platelet aggregation retaining the original scaling of continuous variables to preserve statistical power and to minimize the risk of type II errors. Furthermore, arbitrary cut-off values are overly simplistic and ignore that platelet aggregation is likely to be continuously related to thrombotic risk. Finally, current cut-offs are poorly validated and their use entails a considerable risk of misclassification (101). On the other hand, dichotomization is indeed intriguing to separate "responders" from "non-responders" and may serve as a feasible means to define the potential role of platelet function testing for therapeutic and diagnostic purposes. This was done in the recently published ASCET trial (149), which is the only randomized trial so far to use platelet function testing as a tool to individualize aspirin treatment. For the specific purpose of individualizing treatment, dichotomization seems as a prerequisite, whereas in exploratory studies, like ours, continuous data may be of greater value.

Models for prognostic risk prediction are being increasingly used. In particular, statistically optimal thresholds are often inferred from studies with clinical follow-up by use of receiver operating characteristic curve analyses ("ROC curves") (150;151). However, ROC curves are far from perfect (152), and the complexity of platelet function and antiplatelet drug response makes it unlikely that ROC curve-derived thresholds can serve as the only tool to adjust antiplatelet treatment strategies. Moreover, some risk factors (e.g. smoking) seem to affect the risk of reduced antiplatelet drug response and the risk of adverse events in opposite directions (153;154). Therefore, and because of the limitations of currently available platelet function tests (65), high on-aspirin platelet reactivity (including large areas under the ROC curve) should be regarded as a cardiovascular risk factor rather than a diagnostic marker. Reflecting this, routine measurement of the platelet response to aspirin is not recommended (106).

WHOLE BLOOD COMPOUNDS AND PLATELET AGGREGATION

Study 1 provides evidence that results of whole blood platelet aggregometry depend on platelet count, red blood cells, and white blood cells.

Study 1 extends previous studies on healthy individuals with low platelet counts (155-157) to aspirin-treated CAD patients with platelet counts within the normal range. This is an important distinction, since the vast majority of CAD patients are prescribed aspirin indefinitely and have a normal platelet count. Altogether, our study and previous studies provide valuable information about factors determining the results of platelet function tests. Being a parameter associated with, and partly decisive of, residual platelet aggregation levels, platelet count deserves consideration in any comprehensive evaluation of a platelet function measurement.

It remains controversial to what extent red blood cells affect platelet aggregation (158;159). In our study, red blood cell count and hematocrit correlated inversely with platelet aggregation measured with Multiplate® Analyzer (agonist: collagen) and VerifyNow® Aspirin. This may reflect that red blood cells salvage large quantities of ADP thereby reducing the amount of ADP available to stimulate platelets (160). As discussed in paper I, there are other potential mechanisms, but evidence is scarce.

White blood cell count correlated positively with platelet aggregation levels. This is intuitively plausible given the critical role of inflammation in atherosclerosis, plaque formation, and, ultimately, thrombus formation (161). White blood cells may directly activate platelets by hosting ATPases that reduce ATP to ADP, the latter of which stimulates the $P2Y_{12}$ receptor (162). Furthermore, white blood cells may stimulate platelets through processes mediated by reactive oxygen species including hydrogen peroxide (H2O2) and cathepsin G, a serine protease stored in white blood cells (163;164). Of note, stimulation by reactive oxygen species is enhanced substantially in the presence of platelet agonists such as arachidonic acid and collagen (163;164). Study 1 indeed supports that white blood cells contribute to platelet aggregation, but it does not provide biological explanations for this association.

In study 1, correlations between platelet aggregation and whole blood compounds were only weak to moderate. This implies that the variation in platelet aggregation being directly attributable to variations in platelets, red blood cells, and white blood cells is modest. Moreover, it implies that several other factors influence platelet aggregation, some of them probably much more than the variables measured in our study. Of note, with a few exceptions red blood cells and white blood cells were all in the normal range, and repeating our analyses on patients with more extreme values might yield different results.

ASPIRIN IN PATIENTS WITH PREVIOUS STENT THROMBOSIS

MI is most often induced by superimposition of a thrombus on existing CAD (i.e. atherosclerosis). Generally, ST is considered a distinct cardiovascular phenotype attracting particular attention due to its prognostic severity and the pathophysiological mechanisms causing its occurrence. In studies 2 and 3, we decided to focus specifically on the ST phenotype to explore if ST patients share certain characteristics in terms of platelet function and antiplatelet drug response. At the same time, we acknowledge the considerable similarities between the mechanisms causing platelet dependent thrombus formation in a stented coronary artery and those causing thrombus formation in a non-stented coronary artery.

Studies 2 and 3 revealed significant differences supporting the notion that the ST phenotype is associated with distinct platelet characteristics compared to stable CAD patients and healthy individuals. It is conceivable that a reduced antiplatelet effect of aspirin has contributed to ST formation in these patients, but such causal relation cannot be inferred from our studies. In support of our findings, two other studies reported increased platelet aggregation in patients with previous ST (127;165), but only one of them was restricted to aspirin mono antiplatelet therapy (165). Furthermore, in a prospective study Gori *et al*. identified high platelet reactivity as an independent predictor of incident ST in PCI-treated patients on aspirin and clopidogrel (126). Comparing with the recent ADAPT-DES study, however, high on-aspirin platelet reactivity does not seem to translate directly into increased risk of ST, MI or death (151). The ADAPT-DES registry, a prospective, multicenter study involving >8,500 patients, showed that high platelet reactivity (>550 Aspirin Reaction Units measured with VerifyNow® Aspirin) was not associated with ST one year after PCI (151). Of note, in this study all patients were on dual antiplatelet therapy.

24-HOUR ANTIPLATELET EFFECT OF ASPIRIN

Study 3 is the largest study so far to address the 24-hour antiplatelet effect of aspirin in patients with previous ST. Our motivation to initiate this study was based on the distinct platelet function phenotype of ST patients observed in study 2 coupled with accumulating evidence of reduced antiplatelet effect of aspirin during the standard 24-hour dosing interval (48-53).

Henry *et al*. demonstrated that the number of stable CAD patients with residual platelet aggregation increased five-fold from two to 24 hours after aspirin ingestion (50). In accordance with this and other studies (48;49;51-53), the peak and trough effect of aspirin varied substantially in our study as evidenced by increased 24-hour levels of platelet aggregation and COX-1 activity. Extending previous studies, we also demonstrated increased P-selectin levels at 24 hours indicating increased platelet activation. Concordance between residual platelet aggregation and excess platelet activation is biologically intuitive, but the exact link between the two events remains elusive. While platelet activation is an absolute prerequisite for platelet aggregation, activated platelets do not necessarily proceed with irreversible platelet aggregation (166). Reflecting this, activation of glycoprotein IIb/IIIa occurs at lower agonist thresholds than release of alpha-granule content (alpha-degranulation) (167). Altogether, our findings clearly demonstrate that the antiplatelet effect of aspirin is not sustained throughout the standard dosing interval. Yet, although the reported increase in serum TXB_2 was highly statistically significant, mean TXB_2 levels remained below 30 ng/mL, which implies that at least 95% of COX-1 activity was still blocked (168). Moreover, any clinical relevance of our findings remains to be proven. Important questions for future studies to address are: 1) does twice-daily dosing improve clinical outcome in patients with high on-aspirin platelet aggregation?, and 2) is twice-daily dosing particularly beneficial in patients with increased platelet turnover?

These questions are particularly relevant given the failure of dose increments to improve aspirin efficacy (169;170). CURRENT-OASIS 7 was a randomized factorial trial evaluating high- versus low-dose antiplatelet therapy with aspirin and clopidogrel in ACS patients. The dose of aspirin (300-325 mg daily versus 75-100 mg daily) had no effect on the composite endpoint or on secondary ischemic or bleeding-related endpoints (169;170). Therefore, dose increments do not seem to improve the clinical effect of aspirin, but whether twice-daily dosing does so remains unknown. According to www.clinicaltrials.gov, no ongoing studies are designed to test the effect of multiple times daily dosing of aspirin. However, acknowledging previous reports of diurnal variation in platelet function and clinical events (171;172), an ongoing study is exploring the effect of staggered aspirin intake (i.e. bedtime intake instead of morning intake) (NCT00725127).

PLATELET TURNOVER

Maximal TXA_2 suppression shortly after aspirin administration is determined by blockade of peripheral platelet COX-1, which occurs in the gut capillaries before aspirin enters the systemic circulation (38). In contrast, sustained TXA_2 suppression through 24 hours is highly dependent on blockade of COX-1 and COX-2 in megakaryocytes (173). In patients with essential thrombocythemia, in whom megakaryocytopoiesis and platelet production are increased, Pascale *et al*. demonstrated shorter-lasting platelet inhibition by once-daily aspirin caused by rapid platelet COX-1 renewal (174). Moreover, they demonstrated that twice-daily dosing largely overcomes the reduction in platelet inhibition in these patients. These findings strongly support that the antiplatelet effect of aspirin decreases more rapidly in patients with accelerated platelet turnover.

Increased platelet turnover has previously been linked with reduced antiplatelet effect of aspirin (99;175-178). In study 2, we found increased platelet aggregation in patients with previous ST, and we found a trend towards increased platelet turnover in these patients as well. Evaluation of platelet turnover was based on the immature platelet fraction. More recently, we re-analyzed these data and found that the immature platelet count used as a proxy for platelet turnover yielded an even higher difference between ST patients and stable CAD patients (178). This is in accordance with previous findings from our group (99). Given the physiology of platelets, this may reflect that a certain numerical threshold of platelets, rather than a certain fraction, must be exceeded to generate TX levels that are high enough to elicit platelet aggregation. The results of study 2 served as inspiration for the design of study 3, in which an increased platelet turnover in ST patients was confirmed. ST patients displayed higher levels of platelet turnover 1 and 24 hours after aspirin ingestion whether assessed by immature platelet fraction, immature platelet count or mean platelet volume. Importantly, this was not associated with a significantly higher increase in platelet aggregation from 1 to 24 hours after aspirin intake. Comparing with the study by Pascale *et al*., this likely reflects that ST is a less distinct clinical phenotype than essential thrombocythemia in terms of platelet turnover. At moderately increased levels of platelet turnover, as observed in our ST cohort, COX-1 renewal is not increased enough to yield significant dynamics in the 24-hour effect of aspirin.

THROMBOPOIETIN

Thrombopoietin is considered the primary regulator of platelet production. Therefore, in study 3 we measured thrombopoietin to explore whether differences in the activity of the thrombopoietic system may be causative for variations in the 24-hour antiplatelet effect of aspirin. We found that patients with previous definite ST had increased levels of thrombopoietin compared to stable CAD patients and healthy individuals.

Thrombopoietin seems to have pleiotropic effects. Besides stimulating the megakaryocyte/platelet cell-lineage in the bone marrow, thrombopoietin primes platelets to aggregate in response to otherwise sub-threshold levels of agonists (179) and renders reversible platelet aggregation irreversible (20). Moreover, thrombopoietin may potentiate platelet activation and enhance platelet-leukocyte tethering (180;181). Therefore, it is conceivable that thrombopoietin reinforces platelet aggregation, and at increased levels it may aggravate thrombogenesis. Our thrombopoietin data strengthen the impression of a prothrombotic tendency in ST patients, especially when coupled with the increased levels of platelet aggregation and platelet turnover observed in ST patients in studies 2 and 3.

When searching for mechanisms linking thrombopoietin with platelet aggregation, it is important to consider the mechanisms, by which thrombopoietin levels are controlled. The concentration of thrombopoietin is regulated by negative feedback mechanisms driven mainly by platelet mass (182). A second modulator of platelet production may be inflammation, and a recent study from our group reported weak, yet statistically significant, correlations between thrombopoietin levels and inflammatory markers interleukin-6 and high-sensitive C-reactive protein (183).

An important reservation regarding the above hypotheses is that the exact functions of thrombopoietin remain unclear. The

specific stages of thrombopoiesis during which this molecule is principally active have not yet been unambiguously determined and the link between thrombopoietin and platelet aggregation merits further investigation.

ASPIRIN AND PROTON PUMP INHIBITORS

Since 2006, PPI have been suspected to reduce the antiplatelet effect of clopidogrel (93). Inspired by this finding, we performed the largest laboratory study yet to investigate if concomitant PPI treatment influences platelet inhibition in aspirin-treated patients. In study 4, CAD patients treated with aspirin and a PPI displayed higher levels of platelet aggregation and platelet activation than patients treated with aspirin only. Comparisons were performed using multivariable adjustment to minimize the influence of potential confounding factors.

PPI may reduce platelet inhibition with aspirin through lowered aspirin absorption caused by raised gastric pH levels (Figure 3). This mechanism, which implies that aspirin shifts from its neutral to its ionized form, seems biologically reasonable. However, elevations in pH may also increase the solubility of aspirin whereby the reduction in absorption is theoretically counterbalanced yielding a net effect that may be neutral in terms of gastric absorption. This serves as a possible explanation for the fact that results are not uniform across studies investigating the aspirin-PPI interaction.

A cross-over design was used in a recent study (n = 29) showing no pharmacodynamic interaction between aspirin and PPI (184). Of note, this study included only healthy volunteers. There are other studies showing no pharmacodynamic interaction (185;186) and even one study showing enhanced antiplatelet effect of aspirin in PPI-treated individuals (187).

A large registry-based study including 19,925 aspirin-treated patients with a first-time MI supported a clinically relevant interaction (188). Treatment with PPI, but not with antacid H2 receptor blockers, was associated with increased risk of cardiovascular death, MI, and stroke (propensity score-based hazard ratio 1.61, 95% confidence interval 1.45 to 1.79, $p<0.001$). These findings were contrasted in another large registry study reporting a relative risk of 0.96 (95% confidence interval 0.62 to 1.48) in aspirin-treated PPI users versus PPI non-users.

The randomized COGENT trial (96) confirmed that PPI reduce gastrointestinal bleeding complications in patients treated with aspirin and clopidogrel. The trial also provided strong evidence that the reduction in gastrointestinal bleeding is not accompanied by increased cardiovascular event or mortality rates. Of note, the trial was halted prematurely due to lack of funding and thus was underpowered to appropriately address this important matter. The key lesson from COGENT may be that even if PPI reduce the antiplatelet effect of aspirin and/or clopidogrel, such adverse effects are outweighed by a reduction in bleeding events and, presumably, by increased adherence to antiplatelet medications. Supporting this assumption, a recent analysis showed that co-prescription of low-dose aspirin and a PPI turned out cost-effective by reducing gastrointestinal as well as cardiovascular events (87). As suggested by the authors, the cardiovascular benefit was partly driven by increased adherence to aspirin in PPI users.

6. STRENGTHS & LIMITATIONS

STRENGTHS

Particular strengths of our studies are the well-defined inclusion and exclusion criteria yielding homogenous study populations, the highly standardized blood sampling procedures, the restriction to patients on aspirin mono antiplatelet therapy, the employment of a run-in phase to ensure aspirin steady-state, the unambiguous verification of aspirin adherence, and the use of two thoroughly evaluated platelet function tests.

LIMITATIONS

Common limitations of studies 1-4

The non-randomized study designs did not allow for inference of causal relationships between clinical patient phenotypes, platelet aggregation, and platelet biomarkers.

We did not assess platelet aggregation after withdrawal of aspirin (baseline platelet reactivity) as we considered this unethical in patients at increased thrombotic risk. Thus, we do not know if pre-existing platelet hyperreactivity per se (189) accounts for the higher platelet aggregation in ST patients (studies 2 and 3) and PPI-treated patients (study 4). Furthermore, the lack of off-aspirin measurements precluded us from determining the exact drop in platelet aggregation afforded by aspirin.

Although serum TXB_2 is a widely used marker of COX-1 activity, we used no specific off-target control agonist (e.g. ADP or thrombin) to verify COX-1-independent effects of aspirin.

Blood sampling for platelet function and biomarker analyses was performed between early morning and noon. Acknowledging previous reports of diurnal variation in platelet function (171), this might have influenced the results of our analyses.

Study 1

The study included no thrombocytopenic patients and only few patients with platelet counts above the normal range. Therefore, extending our results to patients with abnormal platelet counts must be done with caution.

The control group of aspirin-naïve healthy individuals was small, and results obtained from this group warrant verification in larger cohorts providing more precise estimates.

Study 2

A considerable number of ST patients otherwise eligible for study participation were treated with other antithrombotic drugs than aspirin and thus were not included. This introduced a risk of selection bias and reduced the number of ST patients, which we partially compensated for by doubling the size of the control group.

The inclusion of ST patients treated with two different stent types (bare-metal stents and drug-eluting stents) likely implies different pathophysiological mechanisms being at play. Furthermore, we did not distinguish between subgroups of drug-eluting stents, although the disadvantages of these stents do not necessarily represent a class effect (75). Differences in stent material and design as well as polymer characteristics are also important to consider (81).

We included different types of definite ST (acute, early, late, and very late ST) acknowledging that they may be caused by different pathophysiological mechanisms, including procedural complications (acute and early ST) and arterial remodeling (late and very late ST). In particular, ten ST patients suffered ST within 24 hours of index PCI and procedural components (e.g. stent underexpansion, stent malapposition, false lumen stenting, inappropriate stent size, coronary artery dissection) are likely to have contributed to in-stent thrombus formation in these patients.

Ideally, the technicians performing platelet function testing should have been blinded for patient category (ST, stable CAD or healthy individuals).

Study 3

ST is a low-frequent clinical entity, and due to difficulties in recruiting patients with previous ST the study was not powered to demonstrate increased platelet function recovery in ST patients compared to stable CAD patients and healthy individuals.

Like study 2, study 3 was limited by heterogeneity in terms of stent type (bare-metal stent versus drug-eluting stent) and timing of ST (acute/early versus late/vey late).

Study 4

Despite multivariable adjustment, we cannot rule out that PPI use is a marker of increased cardiovascular risk (involving increased platelet activation and aggregation) rather than the actual cause of increased platelet activation and aggregation.

Ideally, platelet activation and aggregation should be assessed before as well as during PPI treatment (double-blind cross-over design), but this approach was not practically or economically feasible.

We confirmed adherence to aspirin by measuring serum TXB_2, but we did not employ any biochemical measure of PPI adherence. Importantly, PPI non-adherence would tend to bias our estimates towards null.

7. CONCLUSIONS

STUDY 1

In patients with stable CAD, platelet aggregation depended on platelet count and, to a lesser extent, on levels of red and white blood cells.

STUDY 2

During mono antiplatelet aspirin treatment, patients with previous definite ST were characterized by increased levels of platelet aggregation and immature platelets compared to stable CAD patients.

STUDY 3

During mono antiplatelet aspirin treatment, platelet aggregation, COX-1 activity, and platelet activation increased significantly during the 24-hour dosing interval in patients with previous definite ST, stable CAD patients, and healthy individuals. The increase in platelet aggregation did not differ significantly between groups, but patients with previous definite ST were characterized by higher levels of platelet turnover indices and thrombopoietin.

STUDY 4

During mono antiplatelet aspirin treatment, patients treated with aspirin and a PPI were characterized by increased levels of platelet aggregation and platelet activation compared to patients treated with aspirin only.

8. PERSPECTIVES

A "test-and-treat" strategy must be based on two bedrocks: 1) a platelet function test to identify patients at risk for recurrent cardiovascular events and 2) an effective treatment to minimize thrombotic risk in patients displaying high on-treatment platelet reactivity. The concept of individualizing aspirin treatment is intriguing, but currently there is only limited evidence for the benefit of platelet function testing in this setting. Key studies documenting this are ASCET (149) and ADAPT-DES (151). In ASCET (n = 1,001), stable aspirin-treated CAD patients were randomized to continue on aspirin (160 mg daily) or to switch to clopidogrel (75 mg daily). Aspirin non-responders were identified by the Platelet Function Analyzer-100 aggregation test, and patients switching to clopidogrel had a non-significant reduction in the combined endpoint (all-cause mortality, nonfatal MI, unstable angina, and ischemic stroke) compared to aspirin non-responders staying on aspirin (7.7% vs. 13.1%, p = 0.16) (149). ASCET remains the only randomized controlled trial to soundly appraise and define the value of platelet function testing in individualizing aspirin-based antiplatelet strategies. In ADAPT-DES (n = 8,553), high platelet reactivity assessed with VerifyNow® Aspirin (>550 Aspirin Reaction Units) was not associated with ST, MI or death one year after PCI, which indicates that there is not much to gain by offering aspirin non-responders a distinct aspirin treatment regimen (151). A common challenge in clinical studies, including ASCET and ADAPT-DES, is the low event rates making it increasingly difficult to ascertain statistically significant superiority. Therefore, an important consideration is whether platelet function testing should be broadly utilized or confined to a subset of high-risk patients that may derive particular benefit. Reflecting this clinical dilemma, in stable patients dual antiplatelet therapy versus aspirin alone is expected to avert only ST. In these patients, ST occurs at very low rates and, therefore, the greatest effect of tailoring antiplatelet treatment by platelet function monitoring would be anticipated in high-risk ACS patients.

From a clinician's perspective, a test describing the thrombotic risk of a patient is appealing. However, it is important to distinguish between measuring the pharmacological effect of a drug and predicting clinical outcome in patients treated with the drug. By analogy, platelet function tests employing pathway-specific agonists are well-suited for measuring the pharmacological effect of specific pathway inhibitors. Conversely, given the complexity of platelet function and the interdependence of platelet pathways, "global" shear stress-based platelet function tests have been suggested to provide a more clinically meaningful reflection of the overall activation-aggregation cascade (106). Contrasting this notion, the POPULAR study (n = 951) showed that among tests specific for COX-1 inhibition, adhesion- and/or shear stress-based tests (IMPACT-R and PFA-100) were inferior to aggregation-based tests (light transmittance aggregometry and VerifyNow® Aspirin) in terms of predicting clinical outcome after elective PCI (128). Therefore, before adapting routine use of platelet function testing, there is a need for further methodological developments as well as studies evaluating and comparing tests.

Our expectations to the concept of platelet function testing must rest on an appreciation of its inherent limitations. Firstly, most *ex vivo* platforms are restricted to measurements of platelet aggregation, essentially ignoring preceding key events in platelet function such as adhesion, activation, and secretion (Figure 1). Secondly, it is often argued that agonist concentrations, additives (including anticoagulants), and general *ex vivo* conditions imply an artificial system that poorly resembles *in vivo* platelet biology. Thirdly, platelet function testing is being challenged by evolving platforms, especially within the field of genomics. Insight into the platelet transcriptome, proteome, and metabolome as well as posttranscriptional and posttranslational regulation may prove more useful in tailoring therapy than platelet function testing (190).

It is current practice to administer a standardized treatment to a wide range of patients under the assumption that they all resemble the "average patient". The average patient is inferred from clinical trials, in which average risk estimates are reported. An important limitation of this approach is that the average patient is a mere statistical entity (191). Reflecting this, a substantial number of patients must be treated with aspirin to avoid a single thrombotic event (i.e. large numbers needed-to-treat) (34). Therefore, a major challenge is to translate group-level evidence to individual-level benefit in terms of improved clinical outcome (192;193). Applying this to platelet function testing, dichotomizing according to aggregation levels is likely far too simple to precisely decide the intensity of aspirin treatment or whether switching to another drug is preferable. Therapeutic prediction models have been developed to assess individual patients' responses to treatment based on various individual-level metrics (194). Future studies should determine the value of platelet function testing in individualizing antithrombotic therapy when adding test results to models that integrate numerous clinical and laboratory measures.

9. LIST OF ABBREVIATIONS

ACS = Acute coronary syndrome
ADP = Adenosine diphosphate
CAD = Coronary artery disease
COX = Cyclooxygenase
EDTA = Ethylenediamine tetraacetic acid
MI = Myocardial infarction
PCI = Percutaneous coronary intervention
PPI = Proton pump inhibitor
ST = Stent thrombosis
TX = Thromboxane

10. ACRONYMS

ADAPT-DES
Assessment of Dual AntiPlatelet Therapy with Drug Eluting Stents

ASCET
ASpirin nonresponsiveness and Clopidogrel Endpoint Trial

CAPRIE
Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events

COGENT
Clopidogrel and the Optimization of Gastrointestinal EveNts Trial

CURRENT-OASIS 7
Clopidogrel optimal loading dose Usage to Reduce Recurrent EveNTs-Organization to Assess Strategies in Ischemic Syndromes

ISIS-2
Second International Study of Infarct Survival

11. SUMMARY

Aspirin (acetylsalicylic acid) is an antiplatelet drug used to treat and prevent coronary artery disease. Aspirin is used more frequently than any other drug in the world, however it does not inhibit platelet function equally well in all patients. The risk of platelet-dependent cardiovascular events is increased in patients, in whom aspirin inhibits platelet function suboptimally. Platelet inhibition with aspirin can be evaluated by use of modern whole blood platelet function tests.

The overall aim of this dissertation was to identify and describe functions and limitations of aspirin. We used whole blood platelet aggregometry (Multiplate® Analyzer and VerifyNow® Aspirin) as the primary measure of platelet function. To identify biological mechanisms explaining our findings we also measured a number of biological markers, including markers of cyclooxygenase-1 activity, platelet activation, and platelet turnover. All participants (except healthy individuals in study 1) were treated with non-enteric coated aspirin 75 mg once daily during study participation and received no other drugs affecting platelet function. We used serum TXB_2 measurements to verify that all patients were adherent to aspirin.

In study 1, we investigated the association between platelet aggregometry results and platelet count, red blood cell count, and white blood cell count. The study population consisted of 417 aspirin-treated patients with stable coronary artery disease and 21 drug-naïve healthy individuals. We found consistent associations between aggregation and platelet count, red blood cell count, and white blood cell count. In particular, platelet count was an independent predictor of platelet aggregation, although generally associations were rather weak.

In study 2, we focused on patients previously suffering definite stent thrombosis because these patients may be at a prothrombotic state. We compared levels of platelet aggregation and platelet turnover in 39 patients with previous definite stent thrombosis with levels in 78 patients with stable coronary artery disease. We found that patients with previous definite stent thrombosis displayed increased platelet aggregation and had a tendency towards increased platelet turnover.

In study 3, we investigated if the antiplatelet effect of aspirin is sustained through the standard 24-hour dosing interval. We included 50 patients with previous definite stent thrombosis, 100 patients with stable coronary artery disease, and 50 healthy individuals. We found that platelet aggregation increased significantly through the 24-hour dosing interval, and so did cyclooxygenase-1 activity and platelet activation. The increase in platelet aggregation did not differ between groups, but patients with previous definite stent thrombosis had higher levels of platelet turnover indices and thrombopoietin.

In study 4, we addressed an ongoing debate concerning potential interactions between antiplatelet drugs and proton pump inhibitors. In patients with coronary artery disease, we investigated if the antiplatelet effect of aspirin was reduced in 54 patients treated with aspirin and a proton pump inhibitor compared to 364 patients treated with aspirin only. We found increased levels of platelet aggregation and platelet activation in patients treated with aspirin and a proton pump inhibitor.

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