PLATELET AGGREGOMETRY AND ASPECTS OF RESIDUAL PLATELET REACTIVITY IN PATIENTS WITH CORONARY ARTERY DISEASE TREATED WITH ASPIRIN AND CLOPIDOGREL

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2. LIST OF PAPERS

1. Øystein Meen, Frank Brosstad, Hassan Khiabani, Erik Gjertsen, May Ellen Lauritsen, Turid Margrethe Pedersen, Stine Bjørnsen, Nina Malja Schjelderup, Wivi Ameln, Ee Chye Ng, Marianne Wettergreen, Shazia Parveen Siddique, Gunnar Erikssen. No case of COX-1 related aspirin resistance found in 289 patients with symptoms of stable CHD remitted for coronary angiography. Scandinavian Journal of clinical & Clin Laboratory Investigation 2008; 68:185-191.

2. Øystein Meen, Frank Brosstad, Stine Bjørnsen, Turid Margrethe Pedersen and Gunnar Erikssen. Variability in aggregometry response before and after initiation of clopidogrel therapy. Scandinavian Journal of Clinical & Laboratory Investigation 2009; 69: 673-679.

3. Øystein Meen, Frank Brosstad, Knut Liestøl, Gabor Kunszt, Bjørn Bendz, Marianne Wettergreen, Nina Malja Schjelderup, Trine Andreassen, Gunnar Erikssen. Sequential ADP-stimulated light transmission and multiple electrode aggregometry in patients taking aspirin and clopidogrel after non ST-elevation myocardial infarction. Scandinavian Journal of Clinical & Laboratory Investigation 2012; 72: 318-325.

3. EXPLANATIONS AND ABBREVIATIONS

AA = Arachidonic acid

ACS = Acute coronary syndrome

ADP = adenosine-di-phosphate

ADP test HS = ADP + prostaglandin E1; HS = “highly sensitive”

ATP = Adenosine-tri-phosphate

AUC = area under curve

Between–subject variability = inter-individual variability

CABG = Coronary arterial bypass grafting

CAD = Coronary artery disease

CV = Coefficient of variation

GP = Glycoprotein

HPR = High platelet reactivity

LTA = Light transmission aggregometry

MEA = Multiple electrode aggregometry

MI = Myocardial infarction

NO = Nitrogen monoxide

NSTEMI = non-ST-segment elevation myocardial infarction

PCI = percutaneous coronary intervention

PGI2 = prostacyclin

PPP = platelet poor plasma

PRP = platelet rich plasma

Within-subject variability = intra individual variability

STEMI = ST-segment elevation myocardial infarction

4. SUMMARY

Dual Antiplatelet therapy with aspirin and clopidogrel is a cornerstone in treatment of patients with acute coronary syndromes (ACS) and after percutaneous coronary intervention (PCI), and aspirin is established as antiplatelet monotherapy in stable coronary artery disease (CAD). Despite the fact that treatment with aspirin and clopidogrel attenuates platelet activity, thrombotic events still occur, and the terms aspirin- and clopidogrel "resistance" or "non-responsiveness" - denoting inadequate responses to these drugs, have emerged. Before the work on this thesis was initiated, several studies had demonstrated in vitro on-treatment residual platelet activity, and it was hypothesized that patients who respond inadequately to antiplatelet therapy might be identified by routine in vitro platelet function testing. However; partly due to lacking consensus on definitions, methods and terminology, the prevalence and clinical significance of aspirin- and clopidogrel resistance was controversial – and further research was needed. In order to evaluate the prevalence of aspirin and clopidogrel resistance and to explore and compare two different platelet aggregatory methods we performed three aggregometric studies.

In our first study we explored the prevalence of COX-1 related aspirin resistance among 289 patients with stable CAD who were treated with aspirin. For this purpose we performed in vitro testing using arachidonic acid (AA) stimulated light transmission aggregometry (LTA) and parallel measurements of plasma thromboxane B2 (TXB2). These tests were initially performed on two occasions 2-4 weeks apart and prior to coronary angiography. According to LTA aggregation findings, 11 patients showed signs of inadequate aspirin response on at least one of the two occasions. However, none of these 11 patients showed signs of residual platelet reactivity at a third examination. In conclusion, none of our patients presented persistent COX-1 related aspirin resistance.

In the second study we wanted to explore between- and within-subject variability in ADP-stimulated aggregation before and after initiation of clopidogrel therapy. For this purpose, we performed ADP-stimulated LTA in the 79 of the 289 patients from our first study who underwent PCI and consequently were treated with clopidogrel in addition to aspirin after the procedure. ADP-stimulated LTA was performed on two occasions before

- and one after – initiation of clopidogrel therapy. As expected, large between-subject variability in aggregation was found both before and during treatment with clopidogrel. Besides, there were substantial within-subject variations over time before clopidogrel treatment and in clopidogrel naïve controls. Although the correlation between pre- and post clopidogrel aggregation was significant, reliable predictions of aggregometry responses during clopidogrel therapy could not be made based on pre-treatment testing.

Not only the substantial between-subject variability in ADP-stimulated LTA during clopidogrel therapy, but also within-subject variability in aggregation over time before clopidogrel, suggested that clinically significant within-subject variations in on-treatment aggregation over time might be present. This merited further studies, and in addition we wanted to compare the relatively slow LTA method with the much faster Multiplate Electrode Aggregometry (MEA) method. In our last study we therefore performed repeated, parallel ADP-stimulated LTA and MEA aggregation measurements with 3 different ADP-concentrations in order to explore the agreement between the two methods. The patient population consisted of 31 patients who were on dual antiplatelet therapy with aspirin and clopidogrel after NSTEMI and were treated with PCI; aggregometry was performed on three occasions 6 weeks apart, the first at the time of PCI. As aspirin-and clopidogrel treatment aims to inhibit arterial platelet reactivity while blood sampling acquired for aggregation measurements usually are venous; we also wanted to compare LTA and MEA aggregation in venous and arterial blood.
We found that despite substantial between-subject variability, on-treatment aggregation was quite stable over time in most patients when assessed by both LTA and MEA, and that the agreement between LTA and MEA was good and stable. ADP concentrations had impact on both MEA and LTA assessments, and should therefore be taken into consideration when comparing results based on different ADP concentrations.
We also found that parallel testing in arterial and venous blood showed similar aggregation results both for LTA and MEA – suggesting that venous blood sampling is sufficient for LTA- and MEA assessments.

5. INTRODUCTION

5.1 THROMBOSIS FORMATION. ASPIRIN AND CLOPIDOGREL

ENDOTHELIUM AND PLATELETS: Platelets are small anuclear cells produced in the bone marrow by the megacaryocytes (1) and have an important role in haemostasis to detect and repair vascular injuries, as described 130 years ago by G. Bizzozero (2). Platelets circulate passively as they traverse a vascular tree lined by an intact monolayer of endothelial cells. However; when exposed to vessel wall injury they instantly undergo the process of 1) adhesion, 2) shape change, 3) secretion and 4) aggregation resulting in the formation of a localized haemostatic plug as further outlined below. The interaction between platelets and vascular endothelium is essential for maintaining haemostasis (3). The endothelium consists of a single layer of cells which covers the luminal surface of the vessel wall. Under normal conditions the endothelium acts as a physical barrier that prevents the blood from coming into contact with sub-endothelial tissues. If it is disrupted, blood encounters sub-endothelial tissues containing strong platelet activating and platelet binding proteins such as collagen and von Willebrandt factor (vWF). The platelets adhere to the disrupted area by their specific vWF receptor (GP Ib-V-IX) and collagen receptors (e.g. GP Ia/IIa and GP VI) on the platelet surface (fig. 1) (4-8). In addition to passively preventing the blood from coming into contact with the artery wall, endothelium is also a source of several vaso - and platelet active substances such as the vasodilator Nitric oxide (NO) (3;9), and prostacyclin (PGI_2) which acts both as a vasodilator and a platelet inhibitor (10;11). When the endothelium is injured, these local protective mechanisms are disrupted (12;13). An array of excitatory and inhibitory signals bombards the platelet membrane which is equipped with receptors designed to recognize a variety of extracellular agonist molecules translating them into complex responses by means of second messengers which briefly are outlined below

TXA2 RECEPTOR ACTIVATION AND INTRAPLATELET SIGNALLING: The stimulation of platelet membrane receptors at the site of endothelial injury may initiate platelet activation through several pathways. Most often this activation occurs via different cytoplasmic G-proteins which are coupled to the platelet surface receptors (14;15). One important example is activation of phospholipase C (PLC), resulting in e.g.

mobilization of intracellular calcium stores which in turn triggers activation of different intracellular enzymes. One of these enzymes is phospholipase A2 (PLA2), which releases arachidonic acid from the platelet membrane. Arachidonic acid is converted – first by cyclooxygenase 1 (COX-1) and then by thromboxane A2 (TXA2) synthase – to TXA2 - a strong platelet agonist (16), (fig. 2). TXA2 diffuses through the platelet membrane and induces further platelet activation and recruitment through TXA2 receptors on the platelets surface (15) (fig.3). TXA2 is almost instantaneously (T½ approximately 30 seconds) converted to the more stable TXB2 (16). There are several activation mechanisms within the platelet involving a variety of interacting signalling substances including different protein kinases and also the adenyl- (AC) and guanyl cyclases (GC) important for regulation of the amount of cyclic nucleotides (see below) (15;17). Activation of platelets also results in platelet shape change by activation of the cytoskeleton which is important during thrombus formation (15;17). Also, the coagulation system is activated at the site of formation of the haemostatic plug and the membrane of activated platelets acts as an important site for coagulation complexes interacting with platelet activating substances (such as sub-endothelial tissue factor from the injured vessel wall), resulting e.g. in production of thrombin which is a strong platelet activator (18;19). Interaction between platelets and erythrocytes or leucocytes may also enhance aggregation response (20;21) (see below).

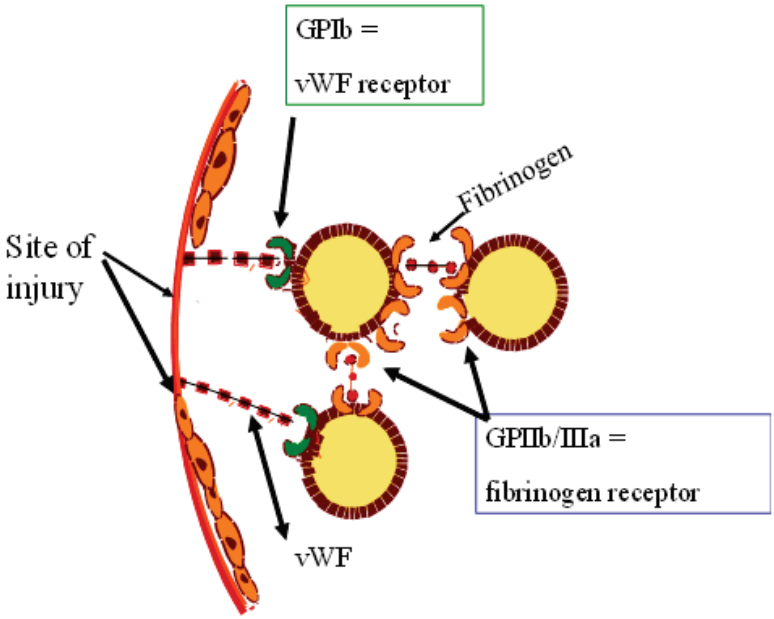

Figure 1: Activated platelets and binding to GP Ib (vWF receptors) at site of endothelial injury and fibrinogen binding (GP IIb/IIIa) receptors interlinking the platelets (Frank Brosstad, UiO/OUS).

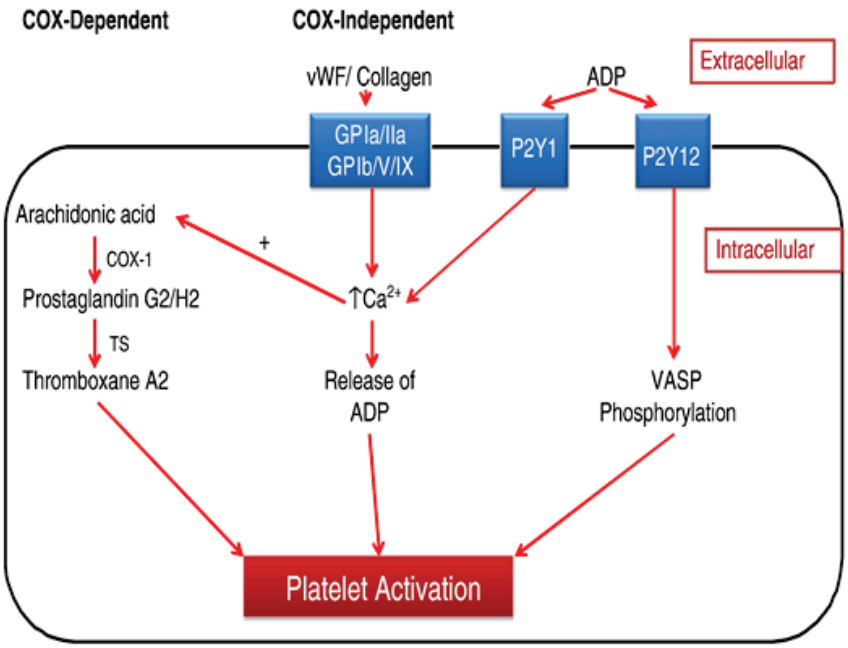

Figure 2: Activation of the platelets by COX-1 dependent pathway and COX-1 independent pathways (R. Fitzgerald et al., Pharmacology & Therapeutics 130 (2011), 213-225).

ADP RECEPTOR ACTIVATION: Not only mobilization of intracellular calcium stores but also influx of extracellular calcium is facilitated during platelet activation. The resultant increase in intracellular calcium promotes platelet release of stored granules (15) containing locally acting pro-aggregatory substances such as the platelet agonist ADP, adhesive proteins (e.g. fibrinogen), and even more calcium ions (fig. 2 and 3). Two different ADP–binding receptors (P2Y1) and (P2Y12) on the platelet surface (14;15), both coupled to intra-platelet G-proteins (fig.2), are important. Stimulation of the ADP P2Y1-receptor promotes, by signalling through the Gq-protein, protein kinase activation. Importantly, stimulation of this receptor also increases the intracellular calcium concentration - which in turn contributes to further TXA2 production (14;15) (fig.2 and 4). Stimulation of the ADP P2Y12 receptor inhibits - through the Gi-protein - cyclic AMP (cAMP) formation (14), and further signalling in turn promotes platelet aggregation by presentation of the GP IIb/IIIa receptors on the platelet membrane (15;22;23), (fig. 4). This inhibitory pathway of adenylat cyclase also facilitates decreased phosphorylation of

vasodilator-stimulated protein (VASP) -which is cAMP-dependent - and in turn promotes platelet activation (24), (fig. 2). The presentation of numerous active GP IIb/IIIa receptors on platelets surfaces is a crucial step in thrombus formation since fibrinogen (circulating and released fibrinogen from activated platelet granules) attaches its two receptor binding ends to two different platelets via their GP IIb/IIIa receptors - interlinking the platelets into aggregates (fig 1) (18;22;23).

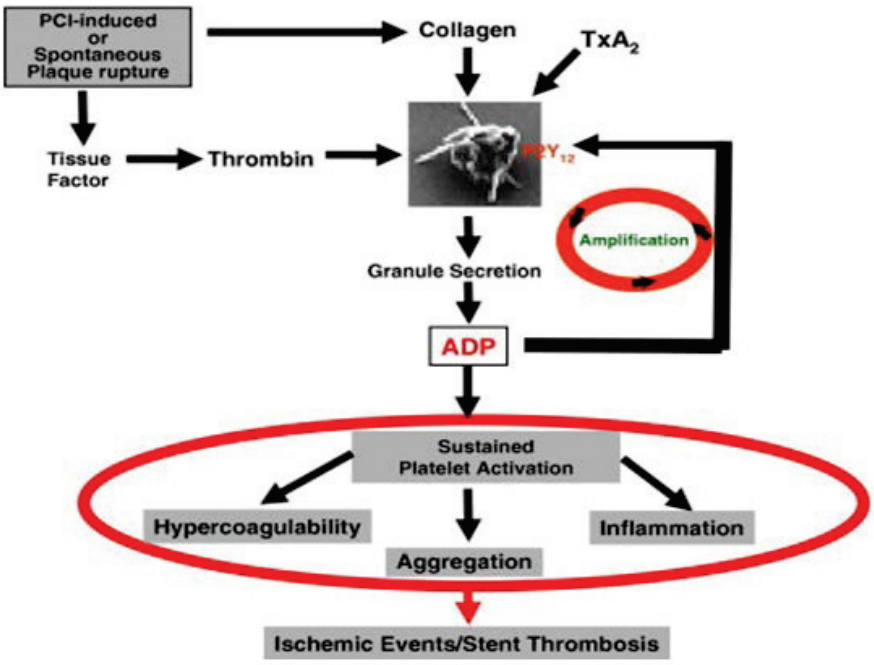

Figure 3: Activation of the platelets by different pathways and the role of ADP (Bonello et al. JACC Vol.56, No 12, sept 14, 2010).

ATHEROMATOSIS/ATHEROTHROMBOSIS: Inflammation plays an important role in atheromatosis and contributes to the development of CAD (20;25-27) in concert with lipids. Dyslipidemia is considered to be the initiating cause of atherosclerosis. The intima of the arterial vessel wall is infiltrated by LDL-cholesterol particles which become oxidized (oxLDL). In response the overlaying endothelium is expressing adhesion molecules that make platelets, monocytes and T-cells adhere and migrate into the subendothelial space by diapedesis (fig. 5). Chemotactic molecules such as MCP-1, IL-8 and RANTES secreted by these cells stimulate to further recruitment.
The monocytes entering the subendothelial space become influenced by macrophage-colony stimulated factor (M-CSF) from endothelial cells to differentiate into lipid macrophages with LOX-1 receptors permitting substantial uptake of oxLDL. Concomitant phagocytosis of platelets turns them into lipid-laden foam cells (28). These cells are prone to apoptosis, releasing their content of oxLDL, chemokines as mentioned above and - perhaps most importantly - matrix metalloproteinases (MMPs) (fig 5). Activated monocytes/macrophages in the plaque area express Tissue Factor that may trigger thrombosis after plaque rupture (29). The T-lymphocytes recognize oxLDL as an antigen which activate them to release pro-inflammatory cytokines that enhance further endothelial and macrophage activation. The cocktail of chemokines also stimulates Vascular Smooth Muscle Cells (VSMC) to proliferate and migrate into the intima, expressing collagen that forms a fibrous cap enclosing the growing plaque (30).
Platelets - apart from their importance in hemostasis - are also potent inflammatory cells that play an important role in plaque formation. When activated they display two important moieties - CD40 and CD40L - also expressed on macrophages, T-cells, SMCs , adventitia cells and endothelial cells- that through CD40-CD40L complex formation allows interaction and crosstalk between these cell types (fig. 6). Platelets may enter the plaque by leaky micro-vessels in the plaque area or by binding to macrophages and T-cells that enters the plaque by diapedesis (fig. 5). Platelet interbonding with the other cell types in the plaque upregulates the expression of MMPs that proteolyse the fibrous cap and make it prone to rupture and subsequent atherothrombosis (31).

ANTIPLATELET DRUGS: The platelets are “inappropriately” activated in arteriosclerosis and contribute to the development of CAD - and if the endothelium is disrupted after e.g. spontaneous rupture of an atherosclerotic plaque or during

percutaneous intervention – the platelets promote formation of an intra-luminal thrombus and eventually ischemic tissue injury (27;32).
It is evident from our knowledge of normal platelet physiology that platelets can be activated in a number of ways, and that several potential targets for pharmacological attenuation of platelet activation exist. Accordingly, several drugs that act through inhibition of one or more of these mechanisms have been developed in order to prevent such "inappropriate" platelet activation. Two of them are aspirin and clopidogrel; the theme of this thesis. Aspirin and clopidogrel have been the most common antithrombotic drugs in use to prevent thrombotic events in CAD patients (33;34) and their widespread use underlines the importance of the platelets in the pathogenesis.

ASPIRIN: Aspirin substantially reduces the risk of myocardial infarction and death in stable coronary disease, and aspirin in combination with clopidogrel prevents ischemic events and re-thrombosis in acute coronary syndromes and after PCI (33;34).
One of aspirin's most important clinical pharmacological effects is to reduce platelet production of TXA2 (35). Platelets are mainly exposed to aspirin as blood passes through the portal circulation in the liver (32;36). Aspirin mediates its platelet inhibitory effect through irreversible acetylation and blockage of the arachidonic acid (AA) binding site on the COX-1 enzyme – thereby inhibiting formation of TXA2 (32;37). This in turn attenuates platelet aggregation and vasoconstriction.

Uncoated (plain) aspirin is absorbed in the stomach, and its platelet inhibitory effect is detectable within 30 minutes and reaches its full platelet inhibitory effect– determined by a decrease in the serum level of TXB2 – within 60 minutes (36;38). As pointed out above, TXA2 is almost instantaneously converted to the inactive, stable TXB2, which can be taken as a measure of TXA2 production (16). Peak plasma level of uncoated aspirin (corresponding to the salicylate level) is reached within 30 minutes (36) after ingestion. Peak plasma level of aspirin after ingestion of enteric coated /controlled release aspirin tablets appears later (39;40) and the platelet inhibitory effect - measured as a decreased serum level of TBX2 – is reached within 6-7 hours (41), to be noted possibly dependent on drug design and release (42). Since blockage of platelet COX-1 is irreversible, aspirin's effect lasts throughout the platelet's lifespan, which is 7-10 days (32). Previous studies have shown that 30 -50 mg of plain (uncoated) aspirin per day is sufficient to inactivate the platelets in healthy subjects and that low-dose aspirin also

gives platelet inhibition in CHD patients (43;44), controlled release aspirin also shows the same accumulative effect (41).

CLOPIDOGREL: Clopidogrel is absorbed from the intestine as a pro-drug which needs to be converted in the liver by the cytochrome P450 enzymes to an active thiol metabolite and this metabolite exhibits clopidogrel's platelet inhibitory effect (45). It is assumed that on the average only about 15% of the absorbed clopidogrel is converted to the active metabolite which reaches maximum serum concentration after approximately 30-60 minutes (46). However, because of variations in clopidogrel's metabolism, and hence the plasma level of the active metabolite the effect of clopidogrel is more difficult to predict than the effect of aspirin.

The active clopidogrel metabolite causes irreversible inhibition of the ADP P2Y12 receptor throughout the platelet lifespan (45;46). Blockage of this receptor promotes, by signalling through the Gi-protein, inhibited platelet secretion of dense granules and ceased inhibition/down-regulation of the adenyl cyclase (AC) (45). These events cause reduced activation of the fibrinogen binding GP IIb/IIIa receptors on the platelet surface (15;22-24;47;48). The combined effect is attenuation of platelet aggregation and adhesion. ADP P2Y12 receptor occupancy of active clopidogrel metabolite is observed as early as 4 hours after ingestion of 75 mg clopidogrel (49). Clopidogrel's maximal platelet inhibition occurs within 4-8 hours after administration of a 600 mg loading dose, somewhat later after a 300 mg loading dose and within 5-7 days following administration of 75 mg/day without an initial loading dose (33;48;50).

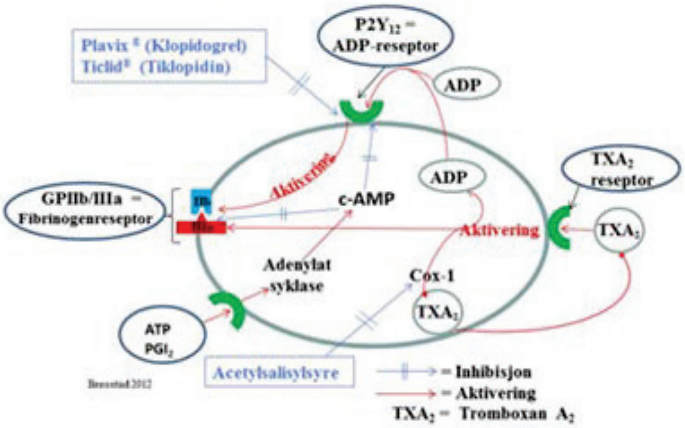

Fig.4 Brosstad 2012

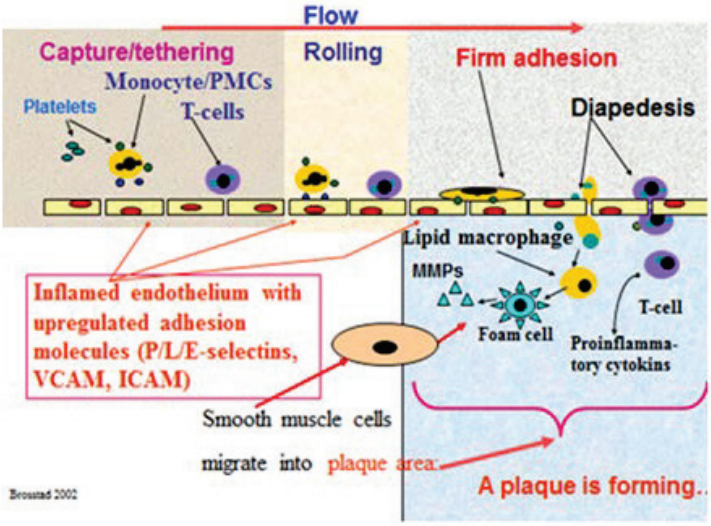

Fig. 5. Brosstad 2012

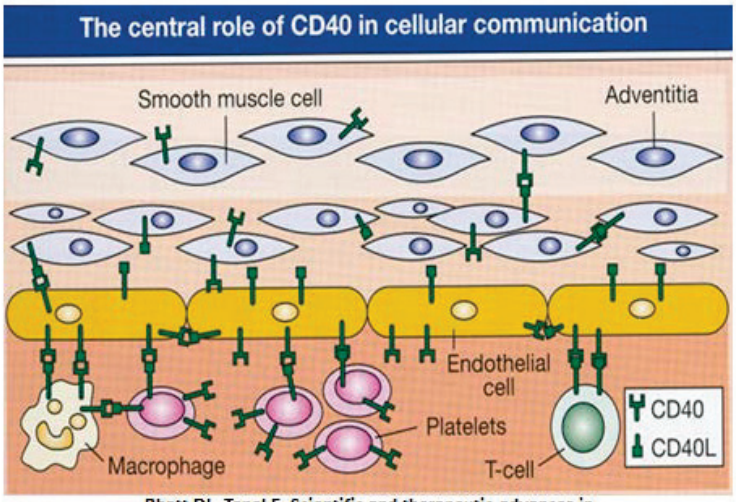

Fig. 6 Bhatt DL. Topol E. Scientific and therapeutic advances in antiplatelet therapy. Nat Rev drug Discov 2003:2:15-18

5.2 ASPIRIN- AND CLOPIDOGREL "RESISTANCE"

Based on both laboratory studies and clinical studies during the past two decades the possibility of inadequate effects of aspirin and clopidogrel in some patients has received substantial attention (51-53). In principle, variability in antiplatelet responses could be related to either pharmacokinetic or pharmacodynamic factors that might affect the bioavailability of the drugs and their action on their targets (47;51;54).

Different terms describing inadequate platelet inhibitory effects of aspirin and clopidogrel occur in the Literature – among which "non- responsiveness" and "resistance" are the most common. Some authors define clinical "resistance" to be present when thrombosis occurs despite antiplatelet treatment. However, because platelets are activated through several mechanisms and aspirin and clopidogrel do not inhibit the entire platelet activation cascade, thrombotic events may obviously occur even when the drugs are effective. Therefore, a better term describing such "clinical resistance" might be "treatment failure" (51). This example shows how discussions of platelet responses to aspirin and clopidogrel have been - and still are, complicated by lack of clear definitions and terminology. In the context of the present thesis we have decided to use the term "resistance" solely when aspirin or clopidogrel might not exhibit their anticipated effects on their pharmacological targets (55).

Several in vitro methods have been used in order to study pharmacological resistance related to antiplatelet drugs. Light transmission aggregometry (LTA) was developed in the early 1960-ies and described by Gustav Born in 1962, and this method, also labeled Born's aggregation (fig.7), is still considered to be the "gold standard" for studying platelet function in vitro (48;55-58). Subsequently, several other methods have been developed. These include e.g. measurements of urinary, plasma and serum TXB2, impedance aggregometry assays as Multiple electrode aggregometry (MEA), Thrombelastography (TEG), Phosphorylation of vasodilator-stimulated phosphoprotein (VASP), PFA-100 and flow cytometry measurements of various pro-aggregatory proteins on the platelet surface (58). In our last study we decided to apply MEA, and to compare this method with LTA.

In order to study possible pharmacodynamic drug resistance, it is essential to use receptor specific methods (55). In case of aspirin this means that methods must be used that can identify insufficient blockage of the arachidonic acid (AA) binding site on the COX-1 enzyme. Since AA is the substrate of COX-1, residual platelet activity should be measured after adding AA in the test tube (48;55). Similarly, in case of clopidogrel, methods should be used that can detect insufficient blockage of the P2Y12 receptor. Therefore, the P2Y12 receptor-agonist ADP should be used as agonist to detect residual platelet activity after ingestion of clopidogrel (48;55) (fig. 2 and 3). In view of these considerations we used AA stimulated LTA in our first study, and ADP stimulated LTA in the second study. In the third study we used ADP stimulated LTA and ADP stimulated MEA in parallel.

6. HYPOTHESES

Study 1

At the time when the work on this thesis was initiated, the debate on aspirin resistance was characterized by lack of a common understanding concerning methodology, definitions and clinical significance. Many studies did not seem to take into consideration that platelet activation occurs by multiple pathways that may act independently of aspirin's action. Importantly, the question regarding whether COX-1 might be incapable of being blocked by aspirin in some patients was unsettled. In order to resolve this issue we specifically wanted to explore possible COX-1 related residual platelet reactivity. For this purpose we decided to use the receptor specific LTA, the "gold standard" method for studying platelet function in vitro. If patients with COX-1 related aspirin resistance might be found that would in our opinion be the "hardest" possible proof of aspirin resistance. Our first study was designed in order to elucidate this question.

Study 2

It was evident from previous studies that between-subject variability in clopidogrel response was substantial even when using methods specifically aimed at detecting blockage of the ADP P2Y12 receptor and that this variability might be of clinical importance. However, we discovered that there was a paucity of data regarding the impact of the variability of the LTA method itself on these findings, and biological within-subject variability in ADP stimulated LTA aggregation over time.
In the second study we decided to explore these possible pitfalls when measuring platelet inhibitory effects of clopidogrel by LTA. We also hypothesized that pre-treatment ADP-stimulated LTA aggregation might predict on-treatment responses to clopidogrel.

Study 3

As evidence regarding the clinical significance of between-subject variability in response to clopidogrel grew, it seemed important to be able to perform in-vitro testing in clinical settings in order to eventually adjust the antiplatelet medication. Classical LTA is time - and labour-consuming. Although several faster tests (like MEA) had been developed, expert opinion was that in order to recommend routine platelet function testing more data were needed. We therefore wanted to compare the performance of the "fast" MEA vs. the

“slow” LTA. Several studies based on single measurements of MEA vs. LTA aggregation had been published, but data on long term on-treatment platelet aggregability and long term agreement between LTA and MEA in several clinical settings were lacking. Both within subject- and between-subject variability of LTA measurements in patients on clopidogrel medication also merited further studies. The primary aims of the third study were to explore within - and between-subject variability in on-treatment MEA-and LTA-aggregation, and to compare repeated, parallel MEA and LTA aggregation over time. We also wanted to explore the impact of different agonist concentrations on the aggregation assessments. As blood samples acquired for testing usually are venous while the platelets play their main role in arterial thrombus formation; we also compared MEA and LTA aggregation in venous vs. arterial blood.

7. MATERIALS

Study 1

The first paper is based on a population of 289 patients with stable CAD on treatment with aspirin who were remitted for coronary angiography. In 270 of the 289 patients AA stimulated LTA was performed in parallel with plasma TXB2 measurements twice 3 weeks apart while on monotherapy with aspirin. In order to obtain reference values for TXB2 we also included a control group of 42 patents without CVD and without medication known to interfere with platelet function.

Study 2

The second paper is based on serial measurements of ADP stimulated LTA aggregation in the 79 patients from Study 1 who were treated with PCI. The first two measurements 3 weeks apart were performed before PCI while the patients were on monotherapy with aspirin, and the last measurement 3 to 52 weeks after PCI while the patients were on dual antiplatelet therapy with aspirin and clopidogrel. A control population consisting of 16 healthy volunteers without medication known to interfere with platelet function was also included in Study 2 in order to provide an estimate of the methodological precision of ADP stimulated LTA at different ADP concentrations (10 µM and 5 µM), and to explore possible sequential aggregometry fluctuations.

Study 3

The third paper is based on three sequential, parallel ADP stimulated LTA and MEA aggregation measurements 6 weeks apart in 31 patients with NSTEMI who were treated with PCI. The patients were on continuous dual antiplatelet therapy with aspirin and clopidogrel. The parallel LTA and ADP aggregation measurements were performed using three ADP concentrations (10 µM, 6.5 µM and 2 µM). Parallel venous and arterial aggregation studies were performed in 10 patients at baseline.

8. LABORATORY METHODS

Light transmission aggregometry (LTA)

As mentioned previously, this method was developed and first described by Gustav Born (fig. 7). The method measures the difference between the light transmission through platelet rich plasma (PRP) prior to and after addition of a platelet agonist (fig.7). Light transmission in platelet poor plasma (PPP) is defined to be 100 % and in platelet rich plasma (PRP) to be 0 % before agonists stimulation. The measured values are expressed as increase in per cent in light transmission compared to the platelet poor plasma plotted against time (definition thresholds for inadequate effect of aspirin or clopidogrel, see chapter 10.1). This method is time- (approximately 2 hours) and labour- consuming and is only performed in dedicated laboratories and university hospitals.

Multiple electrode aggregometry (MEA)

The MEA method is based on the activated platelet's ability - after stimulation by an agonist; e.g. AA or ADP - to adhere to and to form aggregates on electrode surfaces (i.e. metal sensor wires) in whole blood. This testing is performed in anticoagulated whole blood. MEA detects the increase in the electrical impedance resulting from adherence of activated platelets and – aggregates between the two electrodes. The impedance increases proportionally to the number of adhering platelets. The increase in impedance is transformed to "aggregation units" (AU or U) and is plotted against time (definition thresholds for inadequate effect of aspirin or clopidogrel, see chapter 10.1). MEA is substantially less time-consuming (approximately 20 minutes) than LTA.

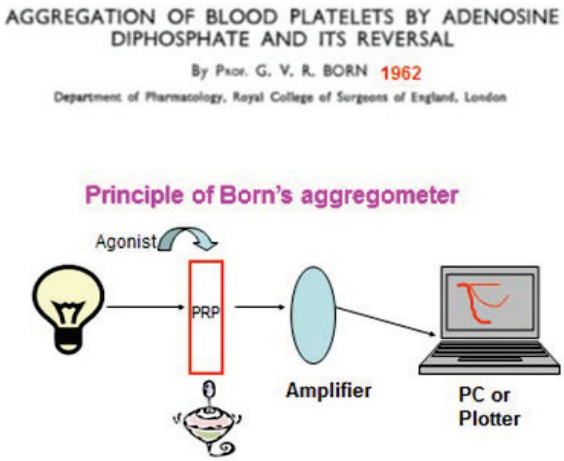

Fig. 7: Principles of Born's aggregometry, first described in 1962 (Prof. Frank Brosstad UiO/OUS)

Plasma Thromboxane B2

Aspirin mediates its platelet inhibitory effect through irreversible acetylation of COX-1 which inhibits formation of the strong platelet agonist Thromboxane A2 (TXA2) from arachidonic acid (AA). As mentioned previously, TXA2 is almost instantaneously converted to the inactive and stable TXB2 - which can be taken as a measure of TXA2 production. An ACE competitive enzyme immunoassay kit (TXB2 EIA kit, Cayman Chemical Company) was applied for measurements of plasma TXB2. Measurements of plasma TXB2 were performed in batches weeks to several months after sampling. Sequential analyses of control samples indicated that there was no drift in TXB2 as a function of storage time.

9. RESULTS

Paper 1

Based on two measurements of AA-stimulated LTA aggregation 3 weeks apart in 270 of the 289 patients (fig. 8), 11 (4.1%) had aggregation ≥20% on at least one occasion. These 11 patients were tested a third time, with aggregation <20% in all eleven before witnessed ingestion of 300 mg of aspirin. Thus, all patients demonstrated an ability to respond normally to aspirin (fig.9).

Average plasma TXB2 level was, as expected, considerably higher in the control group (mean 173 pg/mL, range 8-788 pg/mL) than in patients who were taking aspirin (mean 19 pg/mL, range 1-181 pg/mL) (fig.10). However, the overlap was substantial and taking 45 pg/mL as the TXB2 cut-off level, sensitivity and specificity for detecting patients taking aspirin was 90% and 89%, respectively.

Although their aggregometry response was the same, plasma TXB2 was significantly higher in patients taking aspirin 75 mg daily compared to patients taking 160 mg (fig.10). In the eleven patients who were tested a third time, plasma TXB2 was significantly higher before than after witnessed ingestion of aspirin – despite the fact that their aggregometry responses were the same (fig.9).

We conclude that pharmacodynamic aspirin resistance must be rare. However, our data suggest that some patients may – at least temporarily – require higher and /or more frequent aspirin dosage to achieve the desired antiplatelet effect.

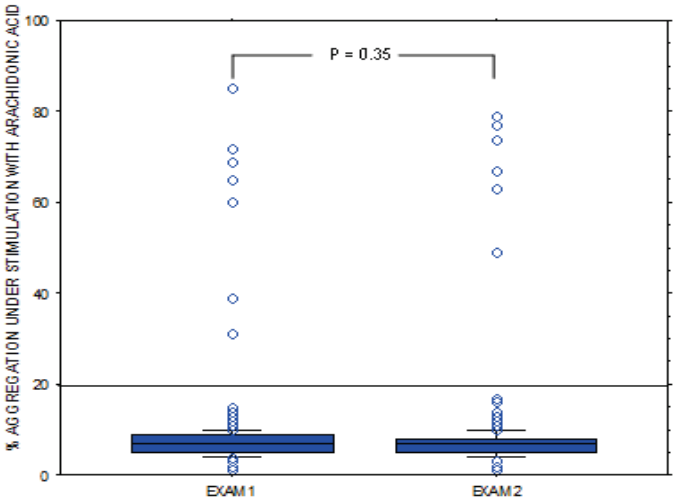

Figure 8: Box plot of arachidonic stimulated platelet aggregation in the first study; exam 1 (289 patients) and exam 2 (270 patients) (paper 1).

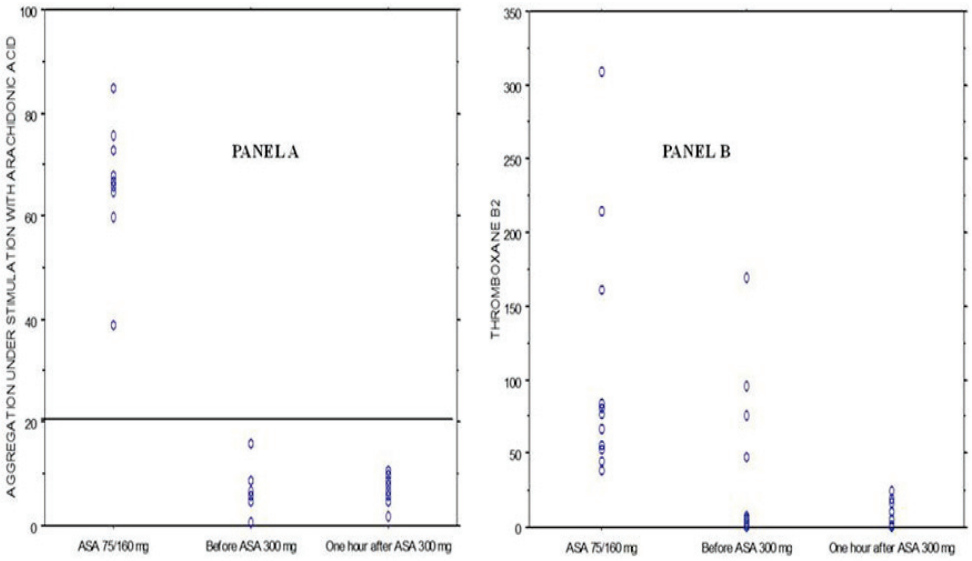

Figure 9: Arachidonic acid stimulated platelet aggregation (panel A) and plasma thromboxane B2 (TXB2) in possible aspirin non-responders (panel B) (paper 1)

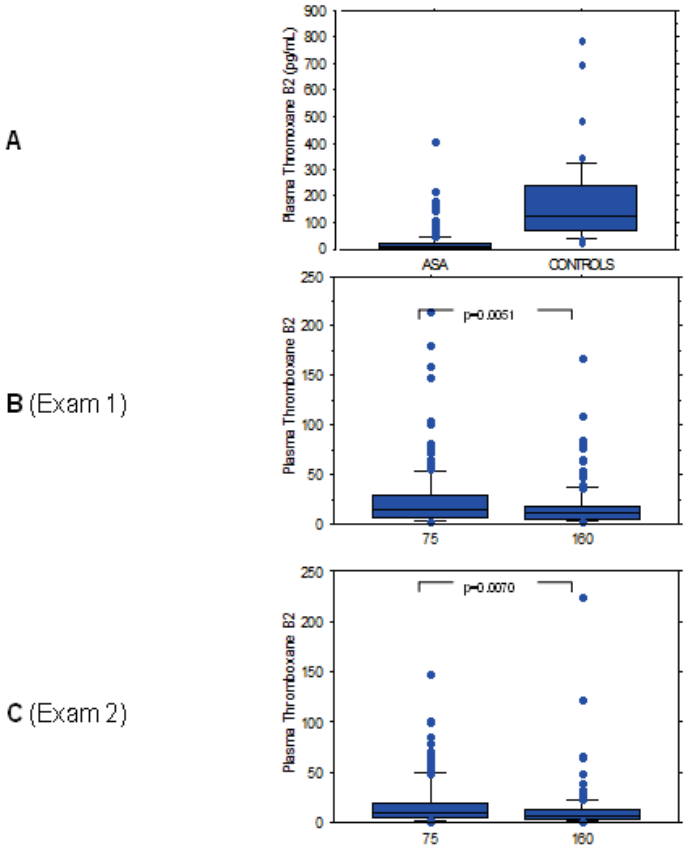

Figure 10: Box plots of plasma Thromboxane B2 (pg/mL).

Panel A: Aspirin (ASA) group (left side; 289 patients on a daily dose of 75 or 160 mg enteric coated aspirin) and in the control group (right side; 42 patients not taking aspirin).

Panels B (Exam 1) and C (Exam 2): Patients taking 75 mg (left) and 160 mg (right). All plots show the median value and the 10th, 25th, 75th and 90th percentile. Observations below the 10th percentile and above the 90th percentile are represented as dots (paper 1).

Paper 2

ADP-stimulated LTA aggregation was measured in the 79 patients who were treated with PCI on two occasions 2-4 weeks apart (Exams 1 and 2) while still on mono antiplatelet therapy with aspirin, and a third time 3-52 weeks after PCI while on dual antiplatelet therapy with both aspirin and clopidogrel (Exam 3). Between-subject variability was substantial (range 17-77%, SD 11.0% during Exam 1), and within-subject changes between Exams 1 and 2 were significant (range -27% to +36%, SD 14.6%, $p<0.05$). Between-subject variability was even larger during Exam 3 (while the patients were on dual treatment with clopidogrel and aspirin) than during Exams 1 and 2 ($p<0.01$). The correlation between aggregations at Exams 2 and 3 was significant, but moderate (RR 0.40). AA-stimulated aggregation was the same before and after initiation of clopidogrel therapy.

In the aspirin and clopidogrel naïve control population (not on antiplatelet therapy) aggregation was stronger and between-subject variability smaller when using ADP 10 µM than when using ADP 5 µM (fig.11). Measurement error was about 6% at both ADP concentrations.

The main conclusions in paper 2 are that since between-subject variability and within-subject variability over time was substantial, prediction of aggregometry responses to clopidogrel based on pre-treatment tests is unreliable. We also conclude that comparisons of aggregometry responses should be performed with caution especially if ADP concentrations are not standardized.

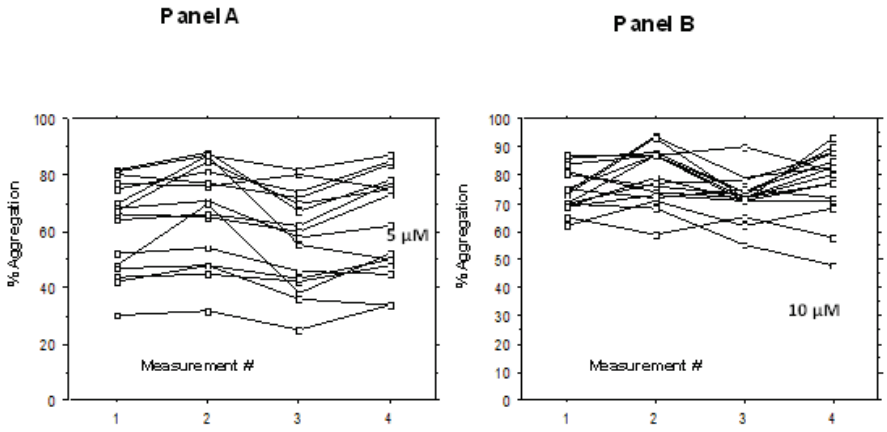

Figure 11: ADP-stimulated platelet aggregation in 16 healthy subjects using 5 µM ADP (panel A) and 10 µM ADP (panel B). In each subject, blood from the same sample was analysed 4 times at each concentration of ADP. Data from the first of two examinations 3 weeks apart, see text (Paper 2)

Paper 3

We found only minor changes in mean LTA- and MEA aggregation over 12 weeks at any ADP concentration. Higher ADP concentrations were associated with stronger LTA and MEA aggregation and higher between-subject variability (fig.12). Within-subject variability in LTA aggregation throughout the study was 7.8%, 8.2% and 5.4% at ADP concentrations 10 µM, 6.5 µM and 2 µM, and corresponding variability in MEA aggregation was 8.8 U, 9.3 U and 7.9 U.

When using the suggested consensus value of 47 U MEA aggregation as cut-off for HPR in MEA and 47% as cut-off value for HPR according to LTA and using the HPR classification according to LTA as reference, 87.1% of the MEA observations gave

correct HPR classification. Only 6.5% of the MEA vs. LTA observations indicated absence of HPR according to MEA in presence of HPR according to LTA (fig. 13).

Both LTA and MEA showed the same aggregation values in venous and arterial blood.

We conclude that within-subject variability over 12 weeks in both MEA and LTA aggregation in NSTEMI-patients on clopidogrel and aspirin medication was moderate, and that the agreement between LTA and MEA was good and stable over time in most patients. As partly pointed out in paper 2; aggregatory responses are influenced by the concentration of agonist.

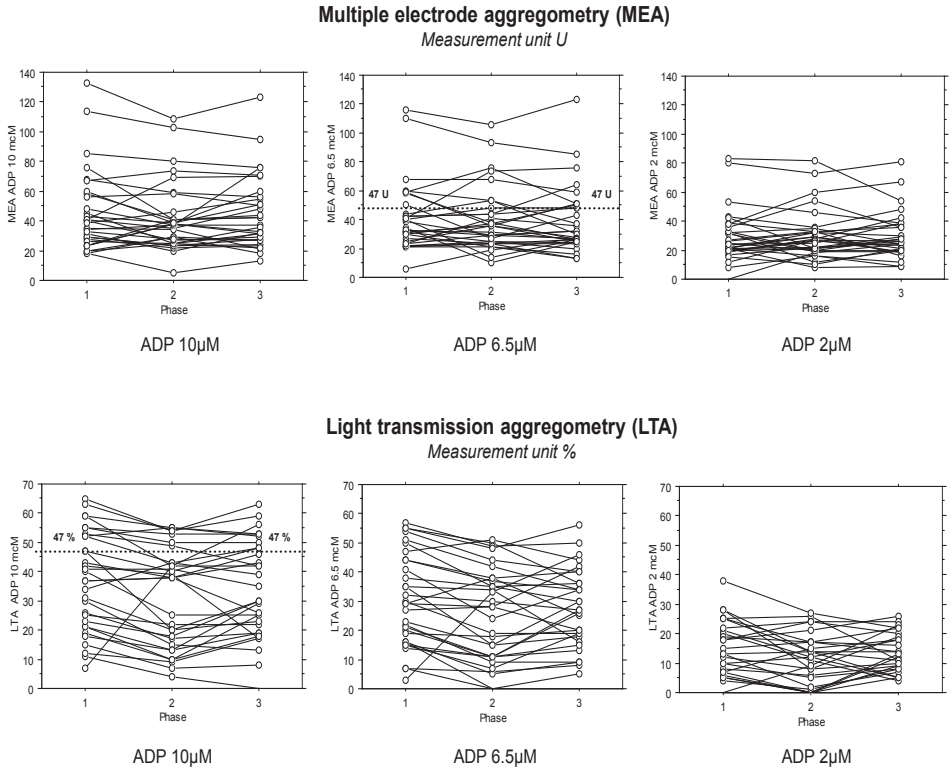

Figure 12: MEA and LTA with 3 different ADP concentrations (2, 6.5 and 10 µM ADP) assessed 6 weeks apart in 31 patients with NSTEMI (paper 3).

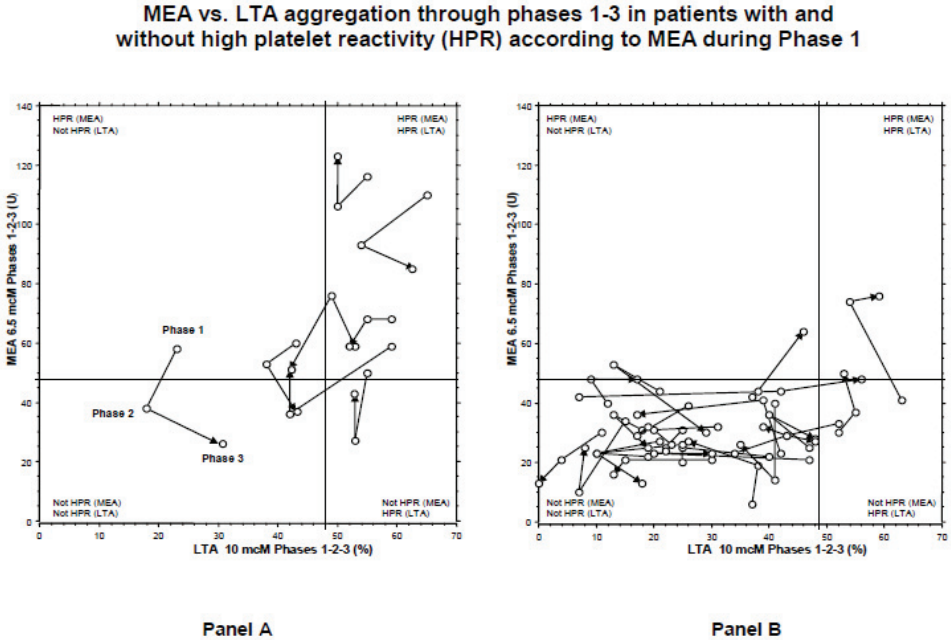

Figure 13. Line chart showing the agreement between MEA and LTA aggregation during phase 1-3.
Panel A: Group with HPR according to MEA (MEA aggregation > 47 U) during Phase 1.
Panel B: Group without HPR (MEA aggregation ≤ 47 U) during Phase 1 (paper 3).

10. DISCUSSION

10.1 ANTIPLATELET EFFECTS OF ASPIRIN AND CLOPIDOGREL. IN VITRO AGGREGATION AND AA STIMULATED LTA, ADP STIMULATED LTA AND MEA

Choice of methods

Aspirin and clopidogrel do not inhibit all pathways of platelet activation. Thus; platelet activation and thrombus formation may still occur - even in the presence of adequate pharmacological effect of these two agents.

Several in vitro methods have been developed in order to determine the effects of antithrombotic medication such as aspirin and clopidogrel (58). These methods are based on assessments of different aspects of the thrombotic cascade and may differ substantially. Residual platelet reactivity may be tested unspecifically by exposing platelets e.g. to collagen, which may induce aggregation despite full effects of the drugs. As a consequence, results from different studies based on different methods are not directly comparable. A crucial aspect in this context is to what extent the methods actually assess the anticipated effect of the specific drug – and consequently their ability to detect a failure to achieve the desired pharmacological response. In our studies we have focused on aspirin and clopidogrel. Therefore, it was essential to use methods that might reveal to what extent the AA binding site on COX-1 was inhibited by aspirin and to what extent the ADP P2Y12 receptor was inhibited by clopidogrel's thiol metabolite (47;48;55) (fig. 2 and 3).

In our studies we decided to use AA- and ADP stimulated LTA in order to detect residual aggregation during aspirin- and clopidogrel medication, respectively, since – as mentioned previously, this method is still considered to be the "gold standard" among in vitro platelet function tests and the LTA is assumed to be less affected by extra platelet COX-1 pathways of platelet activation than several other tests (59). MEA measures aggregation in whole blood, and is one of several alternative methods developed for simpler and less time consuming assessment of platelet function. Previous studies have

indicated that the correlation between MEA and LTA is fairly good. The main differences between these two methods are that LTA takes place in platelet rich plasma while MEA is performed in whole blood which may be considered a more "physiologic environment", and that LTA assesses platelet aggregates in a liquid phase while MEA measures platelet adherence to surfaces.

TXB2 and antiplatelet effects of aspirin

Aspirin irreversibly inhibits both COX-1 and COX-2 (37), but at low aspirin doses this inhibition almost exclusively relates to platelet COX-1 (36;40). Platelets contain considerably more COX-1 than COX-2 (60). Blockage of platelet COX-1 by aspirin is irreversible, and since platelets are unable to regenerate COX-1, inhibition of TXA2 production lasts throughout the 7-10 days platelet lifespan (32).

After being synthesized, TXA2 is quickly converted to the inactive and stable TXB2 which can be taken as a measure of TXA2 production (16;43). As expected, we found that plasma TXB2 was much lower in patients taking aspirin compared to controls. Interestingly - but not surprisingly, and despite the fact that AA stimulated LTA aggregation was similar, reduction in plasma TXB2 was dependent on aspirin dosage (43). Accordingly, patients taking 160 mg aspirin had lower TXB2 than patients taking 75 mg aspirin. Moreover, plasma TXB2 was further lowered after ingestion of 300 mg aspirin among the 11 patients with possible aspirin resistance. This TXB2 may have originated from platelets or extra-platelet sources. It may be hypothesized that an elevated TXB2 level can indicate a lowered threshold for developing an insufficient platelet inhibitory effect of aspirin. If this is the case, some patients may – at least temporarily – require a higher or more frequent aspirin dosage.

In most papers where platelet inhibitory effects have been studied by assessment of TXB2 production, serum TXB2 – and not plasma TXB2 – has been measured. Therefore, although similar, our data are not directly comparable to these studies. Despite the fact that mean plasma TXB2 was almost 10 times higher in the control subjects (without aspirin medication) than in patients taking aspirin, there was a substantial between-subject variability which caused a reduction in diagnostic power.

Antiplatelet effect of clopidogrel

It is clopidogrel's thiol metabolite that conveys the drug's action by selective inhibition of the platelet P2Y12 receptor(45;46). Human platelets express two ADP receptor subgroups on the platelet plasma membrane that are coupled with G-proteins; the P2Y1 receptor and the P2Y12 receptor (15;61). Stimulation of the ADP P2Y1 receptor promotes activating of protein kinase C (PKC) and platelet shape change, and also signals - through the Gq-protein - calcium mobilization (15;61). Stimulation of the ADP P2Y12 receptor induces - through the Gi protein - secretion of platelet granules and also causes amplification of platelet aggregation by activation of fibrinogen-binding GP IIb/IIIa receptors on the platelet surface (15; 22-24;61). The latter platelet activating mechanisms are inhibited by the binding of clopidogrel's thiol metabolite to the platelet ADP P2Y12 receptor.

Interpretations of AA- and ADP stimulated LTA measurements

AA stimulated LTA is considered to be a robust method for assessing COX-1 inhibition. During interpretation of the LTA aggregation curve both maximal aggregation and late aggregation (i.e. after 6 minutes) have been used. Findings based on these definitions are strongly correlated (62), and in our studies we measured maximal aggregation – which is the most common approach (47). Stimulation with AA showed little or no residual platelet activity among our patients which is in accordance with previous studies (55;63). The AA concentration that was used in our study is the recommended concentration and by far the most common, and LTA aggregation >20% (after AA-stimulation) is usually considered as indication of an insufficient effect of aspirin (48).

Non-responsiveness to clopidogrel has been defined differently in different studies. ADP stimulated LTA aggregation >70% has been used frequently (64-69). Other definitions that have been applied are ADP stimulated LTA aggregation >50%, the upper quintile of LTA (or MEA) aggregation in the respective patient populations, percent decrease in post treatment aggregation compared to baseline (usually Δ10%), or just the absolute difference between baseline and post treatment aggregation values (47). However, the agreement between these definitions may be poor (47;59;62). Interestingly, given the >70% ADP stimulated LTA aggregation as cut-off for insufficient clopidogrel response none of the patients in our studies (paper 2 and paper 3) had clopidogrel resistance. This may indicate that drug compliance in our studies was good.

Clopidogrel response assessed by ADP stimulated LTA and MEA aggregation implies interpretation uncertainty since only the ADP P2Y12 receptors - and not the ADP P2Y1 receptors – are inhibited. To what extent activation of ADP P2Y1 receptors influences interpretation of – and comparisons between - LTA and MEA aggregation measurements is an unsettled issue.

Between-subject differences in ADP-stimulated LTA were substantial and significant among both patients and controls (paper 2) and larger than the measurement error among controls at both ADP concentrations. Under the assumption that the measurement error was similar in the both groups we concluded that the observed between-subject differences reflected true biological variations in the response to clopidogrel.

The concentration of the agonists is important both during MEA and LTA, as high concentrations promote stronger aggregation. This has been clearly demonstrated both in our studies among clopidogrel naïve healthy controls and in on-treatment CAD patients, as well as in other studies (47;70;71). The ADP concentrations that are usually applied in laboratories are far higher than ADP concentrations encountered physiologically, and such high concentrations usually induce a pronounced aggregation (22;57). ADP concentrations >10 µM (e.g. 20 µM which is commonly applied (47)) are probably not advantageous methodologically (71). Thus, in our last study, within-subject variability in both MEA and LTA on-treatment aggregation was lowest at the lowest ADP concentration (2 µM) but did not differ significantly between the two highest ADP concentrations (6.5 µM and 10 µM).

Interestingly, although between-subject variability in on-treatment LTA and MEA aggregation was lowest at the lowest ADP concentration (2 µM compared to 6.5 and 10 µM, paper 3), between-subject variability was highest at the lowest ADP concentration among clopidogrel naïve control subjects (5 µM compared to 10 µM, paper 2). These findings are in concordance with previous studies (71). One likely explanation is that patients on clopidogrel treatment present various blockage of the ADP receptors, which causes a substantial dispersion of the aggregation values. On the other hand, individuals who do not use clopidogrel, present stronger aggregation responses in general, this probably causes the clustering of aggregation values up towards the maximum 100% value. One additional aspect to consider regarding these and other ADP stimulated

aggregation data is the possibility of an inherent variability in platelet ADP response - also with respect to the non-inhibited P2Y1 receptor (14) - which may, at least partly, be independent of clopidogrel metabolism and that this difference is unmasked by the higher concentration of ADP. Genetic differences in the ADP 2PY12-receptor may possibly explain some of these effects (72) but not necessarily all (70;73).

Considerations about ADP-stimulated MEA

In our last study we used 3 different ADP concentrations in LTA and MEA - including the concentration recommended by the manufacturer (6.5 µM) for MEA. We did not add Prostaglandin E1 (PGE1) (=ADPtest HS) in MEA as the manufacturer recommends for making the method more sensitive to clopidogrel since this may also lower the specificity of the test - the latter also according to the manufacturer (Compendium by Andreas Calatziz, Ralph Loreth and Michael Spannagl; "Multiplate platelet function analysis-application and interpretation"). However, ADPtest HS and the "plain" ADP stimulated MEA are strongly correlated and the latter also correlates well with LTA (74). Moreover, the agreement between the "plain" ADP stimulated MEA and LTA is acceptable even when using the same ADP concentration (10 µM) in both assays (68;69;75). 10 µM ADP has been recommended as the standard concentration when assessing clopidogrel's antiplatelet effect using LTA (71). In order to keep anticipated specificity as high as possible according to the manufacturer we used only ADP as agonist for assessment of clopidogrel effect by the MEA assay in our study. We applied the suggested consensus value *>47 U* (47) as the cut-off definition threshold for inadequate clopidogrel response.

10.2 ASPIRIN RESISTANCE OR NON-RESPONSIVENESS

As mentioned previously, aspirin resistance has been intensely debated. However, there seems to be increasing agreement among authors that, when using assays that reflect inhibition of the target COX-1 enzyme as in our study the platelet inhibitory effect of aspirin is predictable and strong in the large majority of patients - with an "all or none" response. Furthermore it is assumed- as also suggested in our study- that a lacking in vitro effect of aspirin most often is associated with non-adherence to medication and methodical aspects; and is not a matter of drug resistance (55;63). Thus, our findings are in agreement with other studies (55;62;63;76).

Using methods measuring general platelet reactivity may show considerable aggregation despite aspirin treatment, which may mirror a high pro-aggregatory status due to acute phase or a general heightened pro-thrombotic situation (63). Treatment failure may occur with aspirin as with other medication, emphasizing that the pathways of platelet activation are complex. Although platelet COX-1 enzymes are inhibited by aspirin in vitro, extra platelet sources of TXA2 may still cause activation of platelets, aggregation and thrombosis. Leucocytes, especially the macrophages, erythrocytes and endothelial cells contribute to TXA2 production both through the COX-1 pathway and by increased expression of COX-2 during inflammation - including atherosclerosis (54;60;63). These cells, which also express COX-2, may – unlike platelets – be capable of COX-1 regeneration and TXA2 production during treatment with aspirin.

Inflammatory states, as e.g. seen in diabetes mellitus, in the metabolic syndrome, in obesity and in acute coronary syndromes, may contribute to a pro-thrombotic situation and a weaker antiplatelet effect of aspirin (63;77). Obesity or high body weight may present lower enteric coated aspirin responsiveness as determined by serum TXB2 and possibly also by AA stimulated LTA (42;78;79). Among the 11 possible aspirin non-responders patients in our first study the CRP level and proportion of diabetics was similar to the rest of the study population. However, as mentioned previously, they had significantly higher TXB2 levels. As shown in our study (paper 1), higher doses of aspirin inhibit overall production of TXA2 to a larger extent than lower doses. It is conceivable that this is caused by a more pronounced inhibition of extra platelet sources of COX-1 mediated TXA2, and also to a certain extent by stronger inhibition of COX-2 (60;63). Several other factors in addition to TXA2 and ADP; - i.e. collagen, thrombin, inflammatory substances, leucocytes, erythrocytes and shear forces contribute to platelet activation despite adequate platelet COX-1 inhibition by aspirin, and possibly contribute to the high degree of “aspirin resistance” shown in some studies (47;54;60;63).

As already pointed out, there is still a possibility that insufficient COX-1 inhibition during treatment with aspirin may occur, at least temporarily. During the first study a total of 11 patients showed, for no identifiable reasons, an insufficient response to aspirin; two patients even on two occasions. Principally, as long as drug-adherence to medication is satisfactory; treatment failure of aspirin may be pharmacokinetic (failure to

achieve adequate drug level) or pharmacodynamic (insufficient inhibition of platelet COX-1 despite adequate aspirin absorption):

PHARMACOKINETIC

- Insufficient bioavailability may occur due to insufficient dosing regimes. Aspirin is de-acetylated at various sites including the relatively alkaline milieu in the small intestine. The low pH in the ventricle protects against de-acetylation and favors absorption of plain aspirin. Bioavailability of plain aspirin is approximately 50% (36). Enteric coated aspirin is released in the upper intestine where the pH is higher and de-acetylation may occur and bioavailability of aspirin could be lowered. Thus, enteric coated aspirin may, especially in the presence of high body weight or CAD, cause insufficient bioavailability as determined by attenuated reduction in serum TXB2 and also AA-induced LTA (42;54;78-80). Differences in dissolution in the intestine between different enteric preparations may also exist and restrict bioavailability (42).
- New platelets are generated at a rate of approximately 10% daily (32). Increased platelet turnover and hence platelet count makes the short exposure time of platelets to aspirin insufficient to achieve inhibition of a sufficient proportion of the platelet COX-1 (81). The immature platelets may contain COX-1 and also COX-2 enzymes which are unexposed to aspirin and thus contribute to TXA2 production (54;60) which may be considered as pharmacokinetic insufficiency.

PHARMAKODYNAMIC

- Competitive but reversible COX-1 binding and inhibition by NSAID's, e.g. indomethacin, prevents acetylation of COX-1 (54;82). When indomethacin is reversibly attached to the AA binding site, it remains un-acetylated since acetylation mostly takes place over a rather short time in the portal circulation due to the short half life of aspirin in serum.

- As far as we know in-vitro aspirin resistance with respect to insufficient inhibition of the COX-1 pathway must only to a very limited extent be influenced by genetic factors (63;72;83).

UNSATISFACTORY DRUG ADHERENCE

Non-compliance to medication is an important issue. To overcome non-compliance in clinical trials, witnessed ingestion is preferable, but is difficult to implement. We had no interviews before the second blood test in Study 1, and only contact by telephone a few days before blood sampling in order to remind the patients of drug adherence. Neither did our study protocol advocate pill counting, which would have been scientifically advantageous. Despite our strong efforts to ensure drug adherence throughout all of our studies, we believe that the 11 patients in our first study were not fully compliant. Our observations are in agreement with other studies (47;55;63;84). Non-adherence to medication represents a continuous challenge in assessments of drug response in a clinical setting. In our final study only 31 patients participated in a rather short period of follow up (12 weeks), including 2 telephone calls and interviews during blood sampling which probably enforced drug-adherence

10.3 CLOPIDOGREL RESISTANCE OR NON-RESPONSIVENESS

Since arterial thrombus formation is a multifactorial process, thrombosis may occur even when clopidogrel works effectively. As in the case of aspirin, non-compliance, under-dosing, obesity, drug-drug interactions, accelerated platelet turnover and increased inflammatory activity are possible causes (47;85). Still, many pharmacodynamic and pharmacokinetic aspects of clopidogrel are different compared to aspirin, and aggregation measurements of clopidogrel's effect show large between-subject variability in contrast to aspirin which shows an almost "all or none" response.

Reduced bioavailability of active clopidogrel metabolite resulting in the substantial variability in clopidogrel response according to aggregation measurements is first of all believed to rely on impaired absorption from intestine and on the clopidogrel metabolizing capacity by the liver P450 enzymes (47;72). Both these factors are highly influenced by genetic factors (47;72;86;87). Clopidogrel metabolism in the hepatic cytochrome P450 system involves several isoenzymes among which especially polymorphisms of the CYP2C19 gene seems to be the most prominent genetic

determinant of clopidogrel response (72;86;87). Another important genetic determinant of clopidogrel's bioavailability is the ABCB1 gene which encodes for the efflux pump P-glycoprotein important for intestinal absorption of clopidogrel and is also of clinical importance (72;86;87).

In a study by Sibbing et al. (88), the plasma concentration of the active thiol metabolite correlated with 5 µM ADP stimulated LTA measurements, and was significantly lower in the few patients with stent thrombosis – underlining the importance of proper clopidogrel metabolism. In a study by Bouman et al. (89) plasma concentrations of active metabolite correlated with in vitro aggregation measurements by the VASP-assay, the Verify-Now P2Y12-assay and 20 µM – but not 5 µM - ADP stimulated LTA. However, the actual amount of P2Y12 receptor occupancy by the clopidogrel metabolite is also important, since the amount of metabolite in plasma not necessarily predicts the extent of binding and inhibition of ADP P2Y12 receptors (49;70) measured by various methods. In the studies by Sollier et al. it was shown that the amount of clopidogrel metabolite occupancy of the P2Y12 receptor correlated with LTA aggregation. Other contributing factors to impaired clopidogrel response might hypothetically be other genetic variations of the platelet ADP receptors, but such factors are thought to be of minor clinical importance (72). Interaction with other medication, especially drugs metabolized by hepatic cytochrome P450, may either enforce or weaken clopidogrel efficiency. Rifampicin and St. Johns wort are know to stimulate metabolism by CYP 3A4 and thereby enhance the platelet inhibitory effect of clopidogrel, while erythromycin, ketoconazole, proton pump inhibitors (e.g. omeprazole and esomeprazole), statins (i.e. atorvastatin) and calcium canal blockers may attenuate the effect of clopidogrel. The clinical significance of these interactions is, however, unclear (90-93).

10.4 LTA VS MEA; METHODOLOGICAL CONSIDERATIONS

Methodological differences between LTA and MEA

As suggested by their names, both LTA and MEA (paper 3) are aggregometric methods. However, they are based on different principles (58), and it is by no means obvious that a good agreement between the two methods would be found:

1. Change in light transmittance detected by a photometer (LTA) is caused by changes in platelet shape and adherence causing formation of aggregates in a liquid phase. During MEA the change in electrical impedance between two electrodes caused by platelet adherence and aggregation on the electrode surfaces is measured. The impedance increases proportionally to the quantity of adhering platelets. Adherence to surfaces is an important aspect of platelet aggregation in vivo, and for this reason it may be claimed that MEA gives a better assessment of residual platelet reactivity. At the bottom line, however, both methods assess activation of platelets fibrinogen binding GPIIb/IIIa receptors which facilitate platelet adherence and a prerequisite for the formation of platelet aggregates.

2. LTA is performed in an artificial milieu and includes centrifugation to obtain PRP, and this process may alter platelet function. MEA is performed in whole blood and the cellular environment remains unchanged - including erythrocytes and leucocytes. This milieu is considered to be more physiological compared to the PRP.

3. During blood sampling for in vitro assessments of both LTA and MEA aggregation, interference with intracellular platelet calcium ion concentrations is inevitable. Calcium is, as previously mentioned, involved in several aspects of platelet function and activation. Citrate is the most commonly used anticoagulant but lowers the calcium ion concentration and may cause reduced fibrinogen binding to the GP IIb/IIIa receptors and thus less aggregation (94) . Although some authors do not find differences between MEA assessments based on hirudin and citrate anticoagulated specimens (69), our MEA assessments were performed in whole blood using hirudin anticoagulation as recommended by the manufacturer. Hirudin, a direct thrombin inhibitor, does not affect

the free calcium concentration in the sample. LTA was performed on blood collected in Vacutainer with sodium citrate.

4. LTA is more time- and labour consuming than MEA, and the analysis is more complicated to perform than MEA - including more procedural steps that may increase the risk of operator dependent errors. Importantly, during our studies both LTA and MEA were performed by experienced laboratory technicians, and risk of errors associated with both procedures was therefore, negligible.

5. LTA aggregation has fixed lower and upper limits (0% and 100%), while MEA has no fixed upper limit. Therefore, the statistical relationship between the two methods is non-linear. Consequently, application of conventional methods for exploring between-test agreement (like Bland-Altman analyses) is not straightforward.

10.5 WITHIN- AND BETWEEN SUBJECT VARIATION OVER TIME IN ADP-STIMULATED LTA AND MEA

Pre- and on-treatment ADP stimulated MEA and LTA aggregation

Our second study showed that the correlation between LTA aggregation before and after initiation of clopidogrel therapy was statistically significant, but weak. These findings are in agreement with other studies. Thus, pre-treatment LTA aggregation should not be used in order to predict a patient's response to treatment with clopidogrel (47).

MEA vs. LTA and timing of on-treatment aggregation measurements

Within subject variability over time in both MEA and LTA aggregation was moderate and similar at all ADP concentrations (variation coefficients 17% and 18%, respectively, using 10 μM ADP). These observations are similar to previous findings over short periods of time in stable CAD patients (95). In our study of unstable CAD patients (paper 3), time between clopidogrel loading dose and the first aggregation measurements was 1 to 4 days, and as also shown in previous studies, the response to clopidogrel as determined by LTA seemed to improve somewhat over time – between exam 1 and exam 2 - but was not statistically significant. The overall agreement between MEA and

LTA during the 12 weeks observation period was good since the great majority (87.1%) of the MEA observations gave "correct" high platelet reactivity (HPR) classification according to LTA.

Accordingly, our data suggest that assessment of antiplatelet reactivity only a few days after initiation of therapy during NSTEMI may not be optimal. On the other hand, it may be appropriate from a practical point of view, and altogether our findings suggest that on-treatment measurements of LTA or MEA aggregation performed at one point in time – even a few days after initiation of clopidogrel therapy, may give a reasonably representative picture of the long term antiplatelet effect of clopidogrel. However, the measurements should be judged with caution.

ADP stimulated MEA and LTA and choice of ADP concentrations

As expected, higher ADP concentrations were associated with stronger aggregation (47;70;71). It is noteworthy that between-subject variability in on-treatment LTA and MEA aggregation increased with higher ADP-concentration (2 µM ADP vs. 10 µM ADP). Thus; comparisons of aggregation measurements are of limited value if agonist concentrations differ. However, the difference between LTA aggregations at 6.5 µM vs. 10 µM ADP was small, and there was no significant difference in 10 µM ADP stimulated MEA vs. 6.5 µM ADP stimulated MEA aggregation. Importantly, between-subject variability in ADP 6.5 µM vs. 10 µM MEA and LTA aggregation was similar. Consequently; if between-subject variability in MEA and LTA aggregation is an indicator of differences in perceived responses to clopidogrel, the 6.5 µM and 10 µM ADP concentrations may be interchangeable. 6.5 µM ADP is recommended as the standard concentration in MEA, while 10 µM ADP has been recommended as the standard concentration in LTA in previous publications (71).

MEA and LTA aggregation in arterial and venous blood

We found that MEA and LTA aggregation in arterial and venous blood was similar. Some theoretical considerations suggest that aggregation in arterial and venous blood samples might be different (96): Different sampling conditions (higher pressure, shorter exposure to foreign surfaces during arterial sampling), different concentrations of platelet active substances in arterial compared to venous blood and assumed higher prostacyclin and nitric oxide production in arterial endothelium may theoretically affect the

aggregation results. To our surprise, only one very recent previous study had compared MEA aggregation in arterial and venous blood (96). Our data confirmed the findings from that study. In our opinion these observations are important, since they indicate that venous blood sampling – which is the most common method, reflects platelet reactivity in arterial blood; the target for antiplatelet therapy in CAD.

10.6 FUTURE ASPECTS OF HIGH PLATELET REACTIVITY ASSESSMENTS

Assessments of residual platelet reactivity and clinical use

Increased platelet reactivity assessed by both LTA and MEA (and other methods) is associated with increased risk of thrombotic events in patients treated with aspirin and clopidogrel (64;66;97-101). Several studies have attempted to define threshold values for in vitro platelet reactivity in patients treated with clopidogrel. However, tailored antiplatelet therapy (e.g. increased dosage) for patients with high in vitro platelet reactivity according to such thresholds does not necessarily reduce the risk of thrombotic events (102). Thus- although e.g. a doubled maintenance dosage of clopidogrel is associated with reduced ADP stimulated LTA aggregation and particularly in patients with high platelet reactivity (103)- no studies have so far shown that routine assessments and dosage adjustments are associated with improved outcome.

In vitro assessments of platelet inhibition are test-specific, and different methods do not necessarily identify the same individuals with low drug responses (62;65;67;69;74-76). Interestingly, in our last study (paper 3) almost 90% of the LTA vs. MEA aggregation measurements gave the same high vs. low on-treatment platelet reactivity classification, indicating that the agreement between LTA and MEA was fairly good. However, at the bottom line the clinical significance of all in vitro methods must be evaluated in clinical studies that are aimed at exploring associations between high on-treatment residual platelet reactivity and clinical endpoints. Furthermore, “tailored” therapies guided by in vitro assessments of platelet reactivity must obviously be evaluated in clinical trials and tested for superiority compared to standard therapies.

Thus, in lack of consensus regarding choice of methods, lack of agreement on cut-off values indicating increased thrombotic risk and lack data demonstrating that altered aspirin and clopidogrel dosing improves outcome, the current expert opinion is that introduction of routine in vitro platelet function testing in clinical practice is premature (47;55).

New treatment guidelines for ADP P2Y12 antagonists in CAD

Over the last years the new oral ADP antagonists prasugrel (TRITON TIMI-38) and ticagrelor (PLATO) have shown promising results and are considered to be more reliable inhibitors of the ADP P2Y12 receptors than clopidogrel and have recently been implemented in treatment guidelines for ACS (33;34).

Prasugrel is a thienopyridine pro-drug which – like clopidogrel - needs to be metabolized to an active metabolite by the hepatic CYP-enzymes. However, the metabolism of prasugrel is less dependent on some of the polymorphisms that restrict the bioavailability of the active clopidogrel metabolite. For this reason, in vivo generation of prasugrel active metabolite is considerably more predictable. Prasugrel is, like clopidogrel, an irreversible inhibitor of the P2Y12 receptors, and peak plasma levels and half-lives are similar. Ticagrelor does not need bio activation to achieve platelet inhibitory effect. This drug directly, and in contrast to clopidogrel and prasugrel – reversibly - inhibits the platelet ADP P2Y12 receptor. Metabolism of ticagrelor also yields an active metabolite (mediated by the CYP-enzymes) that reaches about one third of the parent drug concentration. Maximum platelet inhibition after a 180 mg loading dose of ticagrelor or a 60 mg loading dose of prasugrel is obtained within 2-4 hours, compared to 4-8 hours after administration of a 600 mg /300 mg loading dose of clopidogrel (the 600 mg loading dose of clopidogrel induces faster inhibition of platelet aggregation than the 300 mg loading dose) (33;48;50). Onset of action (measured as 50% platelet inhibition) is obtained after 2-4 ours for clopidogrel compared to 30 minutes for prasugrel and ticagrelor (33). Ticagrelor needs to be administered twice daily, while clopidogrel and prasugrel are given once daily. The roles of prasugrel and ticagrelor in future antiplatelet regimens are important and both have already been implemented in the Guidelines for treatment of ACS (33;34).

11. CONCLUSIONS

Antiplatelet therapy with aspirin and clopidogrel has been a cornerstone in the treatment of CAD, but many patients still experience recurrent thrombotic events. There is according to literature an association between attenuated in-vitro platelet responses; labeled aspirin- and clopidogrel "resistance" or "non-responsiveness", and adverse clinical outcomes. Since this seemed to be an important clinical problem, we wanted explore whether patients with pharmacodynamic aspirin and clopidogrel resistance could be identified by in vitro testing. As platelets can be activated through several pathways, our hypothesis was that receptor specific methods should be used in order to reveal whether aspirin and clopidogrel are unable to hit their pharmacological targets. Accordingly, we performed AA stimulated LTA to detect insufficient inhibition of the COX-1 enzyme (the pharmacological target for aspirin) and ADP stimulated LTA and MEA to assess inhibition of the ADP P2Y12-receptor (the pharmacological target for clopidogrel).

In our first study including 289 patients with stable CAD remitted for coronary angiography, we found that aspirin was capable of inhibiting COX-1 in all patients. Accordingly, pharmacodynamic non-responsiveness to aspirin must be rare.

In the second study among 79 patients with stable CAD treated with PCI we found - as expected- considerable between-subject variability in ADP stimulated LTA aggregation both before and during clopidogrel therapy. These between-subject differences were significantly larger than the LTA measurement error, indicating that the differences reflect true biological variability. Moreover, there were significant within-subject variations in pre-treatment ADP aggregation over time, and although there were significant correlations between pre- and on-treatment aggregation values prediction of on-treatment ADP aggregation based on pre-treatment findings may be unreliable and should be done with caution.

In the third study based on 31 NSTEMI patients treated with aspirin and clopidogrel we found substantial between-subject variability in on-treatment ADP stimulated LTA aggregation – similar to our findings in our second study among stable CAD patients.

Findings based on MEA were similar. Moreover, between-subject variability in both LTA and MEA aggregation was highly dependent on ADP concentration; i.e. higher ADP concentrations were associated with larger between-subject variability. Within-subject variability in platelet inhibition by clopidogrel over time determined by both LTA and MEA was moderate at all ADP concentrations, and the agreement between LTA and MEA was fairly good and stable over time in most patients. Accordingly, both LTA and MEA seemed suitable for detecting patients with high on-treatment platelet reactivity. Since we also found that LTA and MEA aggregation was similar in arterial and venous blood, our findings suggest that analyses based on venous samples reflect the situation in arterial blood, and that LTA may possibly be substituted by MEA in similar clinical settings.

Our findings support the current opinion of many experts that failure to prevent recurrent thrombotic events despite antiplatelet therapy is not necessarily caused by pharmacodynamic drug resistance. The term “resistance” should be restricted to a drug’s failure to interact with its target. This may possibly be proper terminology as far as clopidogrel is concerned, but rarely in the case of aspirin. In lack of consensus on both methods of choice and definitions regarding inadequate clopidogrel responses, expert opinion still is that routine in vitro measurements of residual platelet reactivity in order to “tailor” antiplatelet medication is not recommended.

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PAPER I

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PAPER III

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