universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

EVALUATION OF LABORATORY METHODS FOR TESTING ANTI-PLATELET DRUG RESISTANCE

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Martina Leitner
Matrikel-Nummer:	0106183
Studienrichtung /Studienzweig (lt. Studienblatt):	A442 Humanbiologie
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dr. Simon Panzer

Wien, im März 2009

DANKSAGUNG

Vielen herzlichen Dank an Prof. Dr. Simon Panzer, für die Möglichkeit, diese Diplomarbeit zu schreiben und in die spannende Welt der Thrombozyten einzutauchen, für die konstruktive Kritik und die geduldige und herzliche Betreuung.

Weiters gilt mein Dank Frau Beate Eichelberger und Frau Daniela Koren, die mir bei der Arbeit im Labor stets hilfreich, engagiert und mit viel Geduld zur Seite gestanden sind.

Ein Dankeschön möchte ich auch allen Personen aussprechen, die mir für diese Arbeit ihr Blut gespendet haben.

Ganz besonders möchte ich mich bei meiner Familie bedanken. Bei meinen Eltern dafür, dass sie mir ihre Begeisterung für die Naturwissenschaften weitergegeben haben und immer für mich da waren.

Am allermeisten aber, danke ich meinem Freund Tommy, ohne den ich oft nicht weitergemacht hätte, der mit mir durch alle Höhen und Tiefen während des Studiums gegangen ist und besonders in der letzten Phase alles gegeben hat, um mir diese Zeit so leicht wie möglich zu machen.

TABLE OF CONTENTS

ABSTRACT

Impaired platelet function may lead to bleeding in inherited or acquired diseases, but can also be drug-induced and thus helpful to prevent severe thrombotic events such as heart attack or stroke. The response to anti-platelet agents can differ widely between individuals from good to very poor („resistant“). The light transmission aggregometry (LTA) is still considered to be the „gold standard“ method for testing platelet (dys)function, but it has several limitations. Because of the growing interest in testing the efficacy of anti-platelet therapy, new methods were developed with the aim to simplify usage and make them available as point-of-care-instruments.

In this study four whole blood methods were evaluated in comparison to the LTA, focusing on the resistance to the anti-platelet agents Aspirin and Clopidogrel.

Three groups of individuals were tested with the LTA, whole blood impedance aggregometry (WBA, Multiplate®), the vasodilatator-stimulated phosphoprotein (VASP) phosphorylation assay (VASP assay), cone and plate(let) analyzer (CPA, Impact™ and Impact-R™), and platelet function analyzer (PFA-100®, in-vitro-bleeding-time). Normal ranges were obtained from 39 healthy individuals. In 32 cardiologic patients on dual anti-platelet therapy the response to Aspirin and Clopidogrel was tested with arachidonic acid (AA) and ADP (1,6mM and 11µM in the LTA). In the third group (patients with platelet disorders) platelet function was additionally assessed with the agonists epinephrine, ristocetin and collagen.

In reference to the obtained normal values response to Aspirin was found in between 61 to 100% of cardiologic patients. Only the WBA data correlated significantly with LTA data ($r=0,461; p\leq0,01$). A high concordance of test results was achieved between LTA and Impact™ > Multiplate®. Data indicating response to Clopidogrel varied widely between methods (39,1 - 87%). Strong correlations between the LTA data and the Impact™ ($r=-0,793$; $p\leq0,0001$), Multiplate® ($r=0,571$; $p\leq0,001$), Multiplate® HS ($r=0,671$; $p\leq0,0001$) and Impact-R™ ($r=-0,546$; $p\leq0,003$) were found. Best concordance in response was found with Impact-R™. Impact™ and Impact-R™ were only correlating significantly

with ADP (r=0,790; p≤0,0001). The highest frequency of abnormal data was seen with the PFA-100® in patients with suspected bleeding disorders. Multiplate® and LTA obtained data were similar in these patients.

In conclusion, our data suggest that in comparison with the LTA, WBA and CPA are the most adequate methods for testing anti-platelet drug response.

ZUSAMMENFASSUNG

Eine Beeinträchtigung der Thrombozytenfunktion kann bei ererbten oder erworbenen Krankheiten zu Blutungen führen, sie kann aber auch gezielt durch Medikamente herbeigeführt werden und dadurch bei der Vorbeugung von ernsthaften thrombotischen Erkrankungen wie Herzinfarkt oder Schlaganfall mitwirken.
Das Ansprechen auf anti-thrombozytäre Medikamente kann individuell von gut bis sehr schlecht ("resistent") stark variieren. Die Aggregometrie nach Born (LTA) gilt nach wie vor als "Goldstandard" unter den Methoden zur Messung der Thrombozyten(dys)funktion, unterliegt allerdings einigen Einschränkungen. Zunehmendes Interesse, die Wirksamkeit antithrombozytärer Medikamente zu testen, führte zur Entwicklung neuer Methoden, die durch ihre vereinfachte Handhabung auch als „point-of-care" Geräte einsetzbar sein sollen.
In dieser Studie wurden vier Vollblut-Methoden der LTA gegenübergestellt und, mit Hauptaugenmerk auf das Nicht-Ansprechen auf die Medikamente Aspirin und Clopidogrel, evaluiert.

Drei Patientengruppen wurden mit LTA, Vollblut-Impedanzaggregometrie (WBA, Multiplate®), dem Vasodilatator-stimulated phosphoprotein (VASP) Phosphorylierungs assay (VASP assay), dem cone and plate(let) analyzer (CPA, Impact™ und Impact-R™), und platelet function analyzer (PFA-100®, in-vitro-bleeding-time) getestet. Normalwerte wurden mit Blutproben von 39 gesunden Personen ermittelt. Bei 32 kardiologischen Patienten, unter Behandlung sowohl mit Aspirin als auch Clopidogrel, wurde das Ansprechen auf diese Medikamente mit Arachidonsäure (AA) und ADP (1,6mM und 11µM in der LTA) getestet. Die dritte Gruppe (Patienten mit Thrombozyten-Funktionsstörungen) wurde zusätzlich mit den Agonisten Epinephrin, Ristocetin und Collagen getestet.
In Bezug auf die ermittelten Normalwerte wurden bei kardiologischen Patienten zwischen 61 und 100% Ansprechen auf Aspirin festgestellt. Eine signifikante Korrelation ($r=0,461$; $p\leq0,01$) zeigte sich nur zwischen den Ergebnissen der LTA und der WBA. Eine hohe Übereinstimmung der Ergebnisse wurde

zwischen LTA und Impact™ > Multiplate® erreicht. Diejenigen Werte, die auf ein Ansprechen auf Clopidogrel hindeuten, wiesen eine große Streuung zwischen den Methoden auf (39,1 - 87%). Starke Korrelationen traten zwischen der LTA und dem Impact™ (r=-0,793; p≤0,0001), Multiplate® (r=0,571; p≤0,001), Multiplate® HS (r=0,671; p≤0,0001) und Impact-R™ (r=-0,546; p≤0,003) auf. Die beste Übereinstimmung ergab sich mit dem Impact-R™. Impact™ und Impact-R™ wiesen nur mit ADP eine signifikante Korrelation (r=0,790; p≤0,0001) auf. Bei den Patienten mit Verdacht auf Blutungsstörungen wurde das häufigste Vorkommen anormaler Werte mit dem PFA-100® beobachtet. Die am Multiplate® ermittelten Werte waren bei diesen Patienten den LTA-Werten am ähnlichsten.

Zusammenfassend legen unsere Ergebnisse nahe, dass, verglichen mit der LTA, die geeignetsten Methoden zur Beurteilung des Ansprechens auf antithrombozytäre Medikamente die Vollblut-Impedanzaggregometrie und der cone and plate(let) analyzer sind.

I. INTRODUCTION

Platelets play an important role in primary hemostasis to support bleeding arrest in case of vessel wall damage. Impaired platelet function may lead to bleeding complications in inherited or acquired diseases, but can also be helpful in preventing thrombosis formation, if drug-induced.

Atherosclerotic plaques can activate platelets, which consequently form blood vessel occluding clots (thrombosis) leading to acute complications like heart attack or stroke. In high risk patients anti-platelet drugs, such as Aspirin and Clopidogrel, can inhibit different pathways of platelet function, preventing severe events. The response to these agents can differ widely between individuals, from good response to non-response or “resistance”.

Different methods for diagnosing and monitoring platelet dysfunctions have been developed. In recent years more interest is put on monitoring the efficacy of anti-platelet therapy and measuring platelet activity to detect resistant patients. The wide range of available tests suggests evaluation to determine the appropriate method.

1. PLATELETS AND PRIMARY HEMOSTASIS

(Harrison P, George JN, Gawaz, Silbernagl, Schmidt Thews)

Platelets (thrombocytes) are small, discoid shaped, anucleated cells which derive from megacaryocytes and circulate at between 150 – 450 x 10^9 /l blood in the closed vessel system for about 10 days.

In case of vessel wall damage blood can escape into the surrounding tissue. Through a complex process of thrombocyte-activity and interactions between platelets and soluble plasma components the injured vessel wall can be occluded which consequently leads to stable bleeding arrest. It is classified into primary and secondary hemostasis. Primary hemostasis is characterized through adhesion, activation (including a shape change) and aggregation of platelets leading to formation of a primary platelet-rich clot (white thrombus). In

secondary hemostasis the plug is modified through activation of the coagulation cascade into a consolidated fibrin-rich thrombus (red thrombus).

1. ADHESION

Under normal conditions circulating platelets remain in a dormant stage and do not stick to the endothelial cells of the vessel wall. In areas of damage platelets adhere to the now exposed subendothelial structures in a first contact phase, initialized by the release of von Willebrand factor (vWF) which is secreted by the subendothelium. The platelet membrane bound receptor for the vWF (glycoprotein (GP) Ib-V-IX) recognizes this released perivascular vWF and allows the adhesion to the vessel wall (Figure 1).

Stabilization of the adhesion is provided through further membrane receptors for collagen, fibronectin and laminin. A rapid first contact and furthermore a quick formation of stable conditions is necessary due to present high shear forces to the vessel wall.

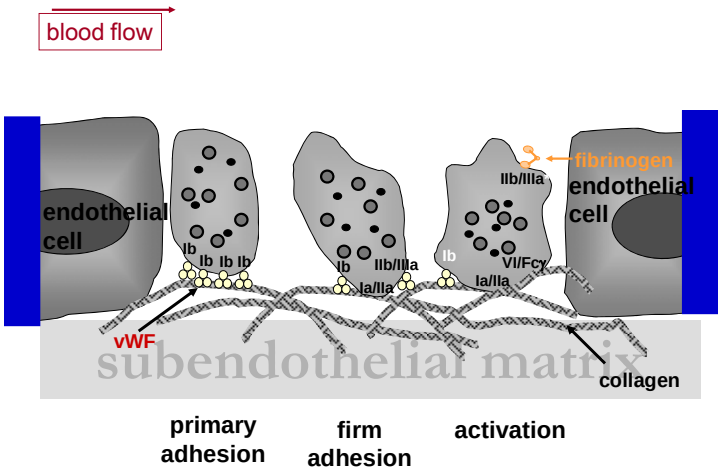


Figure 1: Adhesion and acrivation of platelets in primary hemostasis (with kind permission of Beate Kehrel)

2. ACTIVATION and SECRETION

During the process of adhesion platelets simultaneously become activated (Figure 1). Morphological and functional changes are initialized particularly through binding of the platelet collagen receptor to the subendothelial collagen

and the formation and release of other soluble agonists such as adenosine diphosphate (ADP, induces activation and aggregation) and thrombin (most powerful activating agent) or epinephrine, which bind to their specific platelet surface receptors and induce the formation of second messengers (Figure 2). Once an intracellular Ca^{2+} concentration threshold is exceeded, platelets change into a spherical shape and form pseudopods through polymerization of G-actin and formation of F-actin filaments (shape change). The increased platelet surface supports the interaction of platelets with each other as well as with plasma components and therefore the closure of the damage in the vessel wall.

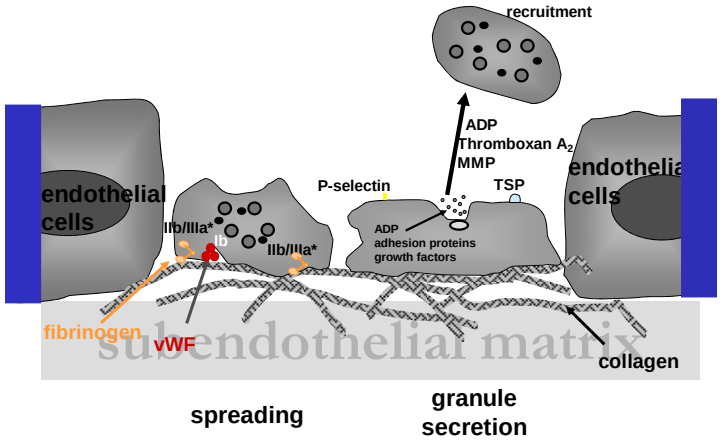


Figure 2: Shape change and secretion of platelets in primary hemostasis (with kind permission of Beate Kehrel)

The secretion of the agonists from secretory granules (dense and α- granules) reinforces adhesion through different mechanisms such as release of Ca^{2+} or the activation of fibrinogen receptors in the GP IIb-IIIa complex. This leads to an activation and recruitment of still resting platelets and a subsequent aggregation with already adhering platelets via GP IIb-IIIa receptors. The GP IIb-IIIa complex also plays an important role in the so called spreading process, which is the final step in adhesion.

Additionally activated platelets produce arachidonic acid (AA) which leads to the formation of thromboxane A_2 (TXA_2). This reinforces the activation process and also acts as a vasoconstrictor and therefore supports clot formation by slowing down the blood flow.

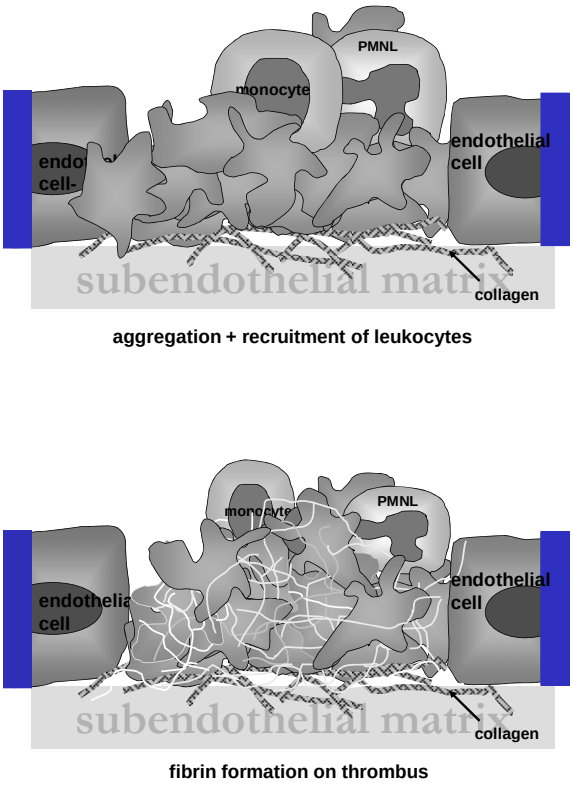


Figure 3: Aggregation and subsequent thrombus formation leading to bleeding arrest (with kind permission of Beate Kehrel)

3. AGGREGATION

Through platelet activation and therefore exposure and activation of GP IIb-IIIa complex on the platelet surface binding sites for fibrinogen get available. The process is strongly dependent on the presence of Ca^{2+} and fibrinogen which are released into the direct surrounding of the growing thrombus. In a first step (primary aggregation) platelets only form loose, reversible fibrinogen bridges (Figure 3). After a time lag of seconds to minutes degranulation of agonists (ADP, TXA_2) ensues, which results in an irreversible connection between platelets (secondary aggregation). When the aggregation process and thus the formation of a platelet-rich thrombus is finished, primary hemostasis is completed. The following activation of the coagulation cascade in secondary hemostasis finally leads to complete bleeding arrest.

Among other functions adhesion, shape change, secretion and aggregation in primary hemostasis can be summarized as “platelet function”.

2. IMPAIRMENT OF PLATELET FUNCTION

1. DRUG INDUCED IMPAIRMENT

Under physiological conditions the hemostatic clot is limited to the injured area and therefore does not affect the blood flow and the supply of the surrounding tissue (Gawaz, 2001). At sites of ruptured atherosclerotic plaques platelets get activated and form clots (thrombosis). Occlusions of the blood vessel through thrombosis blockade the blood flow with subsequent damage of the surrounding tissue and result in acute complications such as heart attack or stroke (Ruggeri, 2002). Platelets not only play a role in the formation of thrombosis but also in the genesis of atherosclerosis (Ruggeri, 2002). There is also evidence that increased platelet activity is determined genetically in patients with coronary disease (Shields et al, 2002).

The multifaceted processes in primary hemostasis open the pharmacological possibility to selectively inhibit pathways through specific targeting (Ruggeri, 2002) for appropriate treatment and prevention of atherothrombosis. Through combination of different anti-platelet drugs inhibiting different pathways, such as the major ADP and TXA_2 pathways, better outcomes can be achieved than with single inhibition (Cattaneo, 2004).

a) Aspirin

The most commonly used agent to decrease the risk of atherothrombotic diseases is acetylsalicylic acid (Aspirin).

i. Mechanism of Aspirin action

The effect of acetylsalicylic acid (Aspirin) is mainly based on the permanent inactivation of cyclooxygenase-1 (COX-1) activity (Roth and Majerus, 1975 ; Patrono et al, 2004) (Figure 4). COX-1 is one of two isoenzymes that catalyzes the transformation of AA into prostaglandin synthetase PGH_2 (Patrono et al, 2004) which is further metabolized to TXA_2 by the enzyme thromboxane synthetase (Fitzgerald and Maree, 2007). The molecular mechanism of the inhibition of COX-1 activity by AA is based on the acetylation of a serine residue (Ser530). Because TXA_2 is largely a COX-1 dependent product, it is very

sensitive to the effect of Aspirin (Patrono et al, 2004) and therefore, if inactivated, ineffective as a vasoconstrictor and for reinforcement of platelet aggregation. Because platelets cannot generate new COX the effect of Aspirin lasts for their life-time (Michelson et al, 2005).

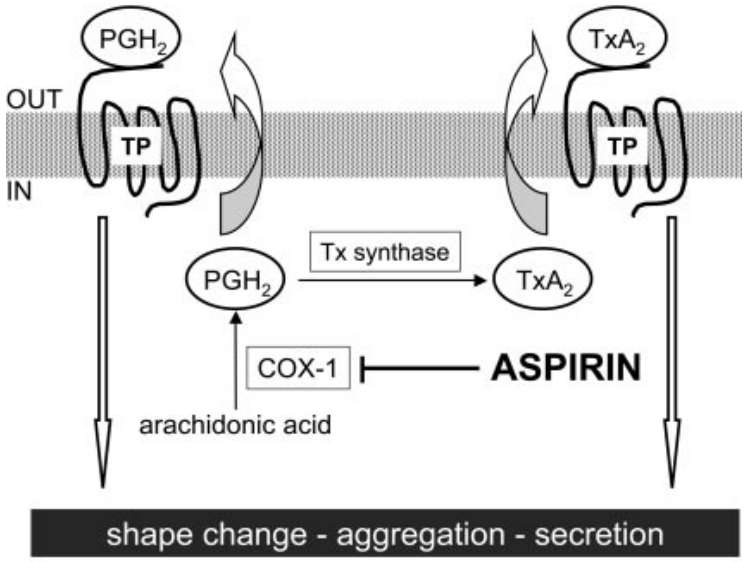


Figure 4 : Schematic illustration of the pharmacological sites of Aspirin action (Cattaneo, 2004)

As platelets are highly involved in acute atherothrombotic events, anti-platelet drugs play an important role in the therapy of linked diseases. In patients with earlier cardiovascular or cerebrovascular events, Aspirin can reduce the risk of a myocardial infarction, stroke or vascular death by about 25% (Antithrombotic Trialist´s Collaboration, 2002). For acute events a loading dose of minimum 150mg are required, whereas for long-term use sufficient anti-platelet effects can be achieved with low doses (75 – 150mg) (Antithrombotic Trialist´s Collaboration, 2002).

b) Clopidogrel

Clopidogrel (Iscover®, Plavix®) is a thienopyridine that irreversibly inhibits platelets and is mainly used in combination with Aspirin.

i. Mechanism of Clopidogrel action

During secretion platelets release ADP from their storage granules which binds to the purinergic receptors P_{2Y1} and P_{2Y12}, therefore reinforcing aggregation at

regions of endothelial lesions (Gawaz, 2001). The ADP-receptor antagonist Clopidogrel, once transformed through hepatic metabolism into its active form (Cattaneo 2004, Gawaz, 2001), inhibits the platelet membrane (Figure 5) ADP receptor P_{2Y12} (Cattaneo, 2004) thereby indirectly inhibiting the binding of plasmic fibrinogen to GP IIb-IIIa complex and thus aggregation (Sharis et al, 1998; Gawaz, 2001). A dose of 75mg per day leads to a significant inhibition of platelets from the second day on and constant levels of activity are found after 4 -7 days (Gawaz, 2001). In a large multicenter trial, where Clopidogrel (75mg/d) was compared to Aspirin (325mg/d) in patients at risk for atherosclerotic vascular disease after stroke, myocardial infarction (MI) or peripheral artery disease, a relative risk reduction for ischemic stroke, MI or vascular death of 8,7% could be found (CAPRIE Steering Committee,1996). It could also be shown that patients with coronary artery disease (CAD) after percutaneous intervention and stenting benefit from inhibition of two aggregation pathways such as through a dual anti-platelet therapy with Clopidogrel and Aspirin (Cattaneo, 2004; Metha et al, 2001; Steinhubl et al, 2002).

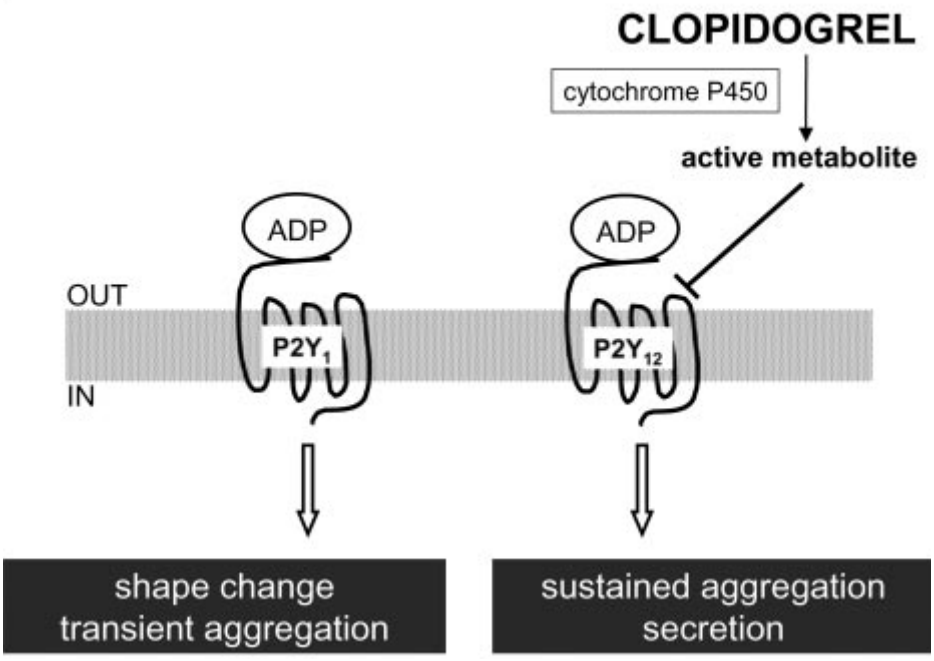


Figure 5 : Schematic illustration of the pharmacological sites of Clopidogrel action (Cattaneo, 2004)

c) Anti-platelet drug resistance

In the last years the existence of „resistance“ or “non-response” to anti-platelet drugs was discussed, because expected effects in patients were not always achieved. This phenomenon is still unclear in many aspects and the evaluation of response to these drugs remains an active field of research (Spectre et al, 2005). Furthermore clarification on the definition of the term “resistance” is needed. As Spectre et al (2005) and Michelson et al (2005) indicate it could be based on whether the clinical outcome or on laboratory test results. Cattaneo (2004) suggests a usage of the term „resistance“ in case of the inability of a drug to hit its pharmacological target because it is unable to reach it or in case of target alteration, but would not consider all patients with atherothrombotic events despite intake of anti-platelet drugs as “resistant”. This “clinical resistance”, defined mainly in connection with Aspirin, should better be termed as “treatment failure” (Cattaneo, 2004; Patrono, 2003; Michelson et al, 2005). It also has to be taken into account that Aspirin and Clopidogrel only inhibit specific pathways, whereas in the genesis of thrombosis many agonists are involved, which makes it impossible for the anti-platelet drugs to prevent all events (Fitzgerald and Maree, 2007).

Based on laboratory tests, resistance to anti-platelet drugs is also not necessarily given when high residual platelet reactivity is found, especially when the tests used are not specific to the effect of the agent. However, non-specific tests are helpful to detect hyperreactivity, although only specific ones can determine whether it is caused by a weak pharmacological effect of the drug or not (Cattaneo, 2007).

Furthermore, as the dosage of drugs is often determined by the population response, selecting a treatment where risk and benefit is balanced will also include individuals who will not respond to the chosen dose (Fitzgerald and Maree, 2007)

i. Aspirin resistance

In patients receiving Aspirin a wide variability in response is found and as different laboratory tests including different cut-off values are used, the frequency of non-responders varies in studies (Cattaneo, 2007). Patrono et al

(2004) report about recurrent thrombotic events in about 10 - 20% of Aspirin treated patients with arterial thrombosis (during long-term follow up). Campbell and Steinhubl (2005) report in a review of 11 studies using non-specific tests that rates varied between 5,5 to 61%. Other studies found resistance in 1 – 1,7% when measuring thromboxane B_2 (TXB_2) levels, or below 1% in case of testing AA induced platelet aggregation, which leads to the assumption that Aspirin resistance appears extremely rare, when using specific tests (Cattaneo, 2007).

Different reasons that are eventually responsible for the non-responsiveness to Aspirin, such as reduced bioavailability or too low dosage respectively, an accelerated platelet turnover, the presence of less sensitive COX-1 variants or transient resistance caused through coronary surgery are summarized by Cattaneo (2007). Furthermore simultaneous intake of other drugs can influence the effect of Aspirin (Patrono, 2003). But the most plausible and most frequent reason for resistance or poor response is lack of compliance as Frelinger et al (2006) describe. Additionally Gurbel et al (2007) found out, that in patients with diabetes a higher dose of Aspirin is needed to achieve similar results as in non-diabetic patients.

As described above it is unclear whether the definition of “resistance” should be based on laboratory measurements. Concerning Aspirin it is possible to determine non-responders by conducting functional tests or biochemical measurement of serum TXA_2 (Spectre et al, 2005). “Resistance” based on early platelet aggregation tests was difficult to interpret considering the fact that platelet aggregation tests have not been found useful to describe the pharmacology of Aspirin (Patrono, 2003). Based on Cattaneos definition (Cattaneo, 2004) Aspirin resistance is given when the COX-1 dependent TXA_2 production cannot be inhibited (Cattaneo, 2007).

ii. Clopidogrel resistance

Although using specific tests a wide variability in response to Clopidogrel was found, including a significant amount of low responders and results of up to about 30% cases indicating non-response (Aleil et al, 2005; Grossmann et al, 2004; Gurbel et al, 2003).

Most likely these differences occur due to variability in the metabolization of the pro-drug caused by genetic variance of the activating enzyme cytochrome P450, whereas polymorphisms in the ADP-receptor seem not to be associated to interindividual differences in response (Cattaneo, 2007; Fitzgerald and Maree, 2007). Furthermore other reasons for interindividual variability could result from interactions with other drugs, low bioavailability through different absorption or wrong dose in relation to BMI, or from already existing differences in response of platelets to ADP (Cattaneo, 2007; Fitzgerald and Maree, 2007). The found variability in response seems to indicate differences in bioavailability and not “resistance” of the ADP-receptor as defined by Cattaneo (Fitzgerald and Maree, 2007; Cattaneo, 2007).

d) Monitoring of anti-platelet drugs

Grumann et al (2007) suggest monitoring and individual adjustment of anti-platelet therapy as a good possibility for optimizing individual inhibition to identify resistant patients and therefore prevent recurrent events or to prevent bleeding through individual overdosing. Some studies have demonstrated that MACE (major adverse cardiac events) in cardiovascular diseases can predicted through platelet function tests, but these assays first have to be studied in large clinical trials before being regularly used in clinical care (Michelson, 2004).

2. INHERITED PLATELET DYSFUNCTIONS

In inherited platelet disorders bleeding occurs varying from spontaneous, mostly mucocutaneous bleeding to excessive traumarelated bleeding in milder forms (Nurden and Nurden, 2008; Keeney and Cumming, 2001)

a) Von Willebrand Disease

The von Willebrand disease (vWD) is characterized through impaired adhesion because of qualitative and quantitative deficiency of the vWF (Ruggeri and Zimmerman, 1987; Platelet Perspectives® http://www.plateletperspectives.com, 2007). About twenty subtypes have been described, the actual classification defines six different types (Ruggeri and Zimmerman, 1987; Sadler, 1994). Type 1 is the most common type of vWD and occurs in 70-80% of all cases

(allaboutbleeding.com; http://www.allaboutbleeding.com, 2008; HUMATE-P, http://www.humate-p.com, 2009). It is associated with partial quantitative vWF deficiency which results in mild to moderate bleeding tendency (Sadler, 1994; Keeney and Cumming, 2001). Type 2 is subclassified into 2A, 2B, 2M and 2N and characterized through a qualitative defect of the vWF (Keeney and Cumming, 2001). In type 2B thrombocytopenia and giant platelets can be found (Nurden and Nurden, 2007). Type 3 is described as a severe quantitative disorder because of very low or absent platelet and plasma vWF (Keeney and Cumming, 2001). Additionally a platelet type is described which is similar to type 2B vWD (Nurden and Nurden, 2008).

All forms of vWD are autosomal dominant in inheritance except type 2N and 3 which are passed on autosomal recessive. As Gill et al (1987) described, a correlation of the AB0 blood group system with the occurrence of vWD was shown.

In rare cases a defect of vWF can also be acquired (acquired von Willebrand syndrome (AVWS)) (Eikenboom et al, 2007, Kumar et al 2003**,** Velik-Salchner et al, 2008). It is mostly associated with underlying diseases such as lympho- and myleoproliferative disorders, malignancies, hypothyroidism, immunologic or cardiovascular diseases, but also induced by drugs and autoimmune diseases (Federici et al. 2000; Velik-Salchner et al, 2008; Federici et al., 2004; Mohri 2003). vWD and AVWS show quite similar laboratory “phenotypes” and bleeding symptoms, which makes it difficult to differentiate in clinical practice (Eikenboom et al, 2007).

b) Bernard-Soulier syndrome and Glanzmann thrombastenia

Bernard-Soulier syndrome (BSS) and Glanzmann thrombastenia (GT) are also inherited diseases where bleeding symptoms occur due to platelet function abnormalities in primary hemostasis, but are found quite rarely. Such patients were not studied.

3. DISEASE ASSOCIATED IMPAIRMENT

Abnormal platelet function can be acquired through anti-platelet drugs as described above but also through different diseases such as diabetes mellitus,

liver disease, immune-mediated diseases or uremia and end stage renal failure (ESRF) respectively. Since only 5 patients with ESRF were included in this study, the other disease-associated platelet function impairments are not further discussed.

a) Platelet function disorder in uremia

In patients with uremia (and ESRF) both hemorrhagic diathesis and thrombosis tendencies can be found (Hörl, 2006). Hemorrhagic diathesis in these patients is associated with both thrombocytopenia and impaired platelet function. Thrombocytopenia, quite likely because of early destruction of platelets (Himmelfarb et al, 1997) is found in 16-55% of uremic patients (Hörl, 2006). However, bleeding tendencies are mainly caused by impaired platelet adhesion and aggregation. Adhesion is reduced because of reduced GP Ib levels (Himmelfarb et al, 1998; Sloand et al, 1991). Though normal levels of GP IIb and GP IIIa are found (Walkowiak et al, 1994), aggregation is impaired through inadequate conformational changes in the GP IIb-IIIa complex, which is caused quite likely because of uremic toxins (Gawaz et al, 1994). Furthermore a functional cyclooxygenase defect and reduced production of TXA_2 were found in patients with renal failure (Remuzzi et al, 1983). Receptors for vWF and fibrinogen can also be affected through inappropriate membranes in dialysis, which leads to interaction defects between platelets and with the vessel wall (Hörl, 2006).

As mentioned above also increased tendencies for thrombosis were found in patients with renal failure. Increased platelet aggregation and hypercoagulation associated with higher levels of erythrocyte-platelet-aggregates (Sirolli et al, 2001) or platelet microparticles (Ando et al, 2002) were found compared to healthy individuals.

The use of anti-platelet drugs to prevent shunt-thrombosis in patients with ESRF is nor recommended as they do not reduce the risk of thrombosis, but rather lead to increased bleeding tendencies (Kooistra et al, 1994; Kaufman et al, 2003).

3. PLATELET FUNCTION TESTING

Many tests have been developed to identify causes of abnormal bleeding and to monitor pro–haemostatic therapy in case of increased risk of bleeding. Apart from these major applications platelet function tests are furthermore used to predict thrombosis by identifying platelet hyperfunction and to monitor the efficacy of antiplatelet therapy (Rand et al., 2003; Harrison, 2005). Due to the large number and variety of platelet dysfunctions there is no current platelet function test that is 100% sensitive (Harrison, 2005).

The oldest way to test platelet function is the in vivo method bleeding time (BT), developed by Duke in 1910 and later modified by Ivy and regarded as the most useful screening test for platelet function until the early 1990s (The British Society for Haematology, 1988). BT is the measured period of time for bleeding to stop after inducing a lesion, usually into the forearm. The test was refined through using specific templates for making standardized cuts (Harrison, 2005), but it is no longer recommended due to its non-specificity and "lack of clinical correlations" (Michelson et al, 2006).

In the following years a series of in vitro methods became available. Besides the light transmission aggregometry (LTA), which was presented in the early 1960s by Born and is still considered to be the "gold standard", new instruments were developed in the last years, with the aim to be simpler in use, available as point-of-care (POC) instruments (and not only in specific laboratories) and less invasive. Platelet function can be tested in whole blood, which requires no more sample preparation, shorter assay times and smaller amounts of blood. Furthermore with some methods platelet function can be tested under close to physiological conditions including shear forces. Additionally the new methods are trying not to be limited to specialized operators and to be more cost-effective.

1. ESTIMATION OF PLATELET FUNCTION IN PLATELET RICH PLASMA

i. LIGHT TRANSMISSION AGGREGOMETRY

Through the invention of the in vitro method LTA in the 1960s the diagnosis of platelet function defects could be notably improved. The method is based on the

measurable change of turbidity in platelet rich plasma when platelet aggregation is induced through agonists (Figure 6). The biggest advantage of platelet aggregometry is that the aggregation of platelets can be measured in vitro in a GP IIb-IIIa dependent manner, which is the most important function of platelets (Michelson et al, 2006).

Different studies have shown that measurement of platelet aggregation with LTA can detect platelet hyperreactivity in healthy individuals (Yee et al, 2005) or predict the risk of recurrent MACEs in patients (Matetzky et al, 2004; Gum et al, 2003; Frere et al, 2007). Furthermore it is an appropriate method for diagnosing inherited platelet function defects (Budde, 2002).

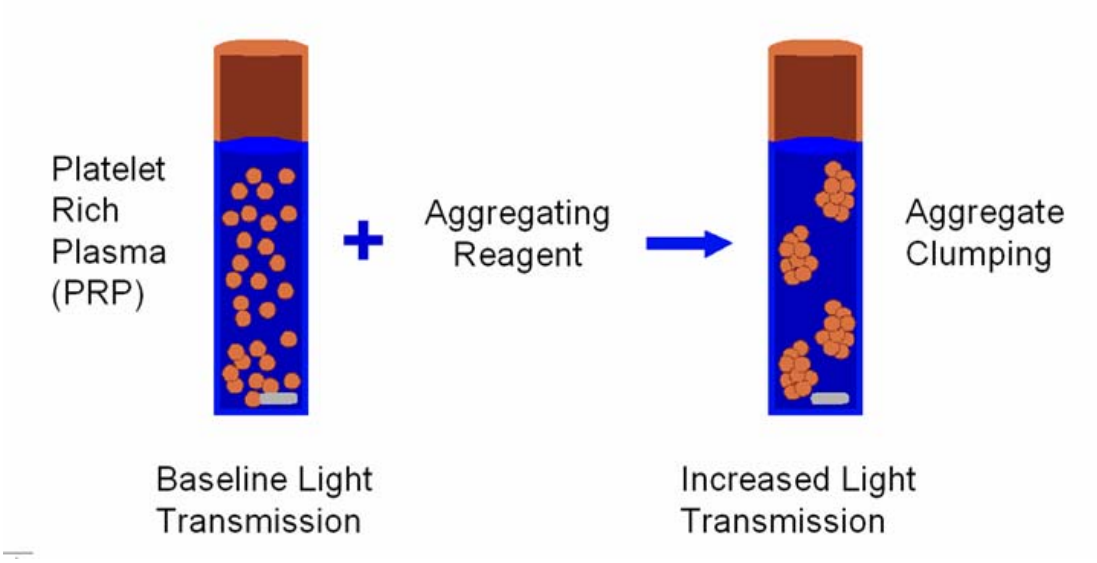


Figure 6: Light transmission aggregometry principle

However, LTA has many disadvantages, as platelets are not in their physiological milieu which includes red cells and leukocytes, the system runs under low shear conditions, and formation of aggregates only after addition of agonists, time consuming centrifugation steps, requirement of large amount of blood samples, requirement of specialized operators, and cost (Harrison et al, 2007; Shenkman et al, 2008; Michelson, 2004). Additionally, there is still a lack in standardization between laboratories including used concentrations of agonists or if platelet-count-adjustment in platelet-rich plasma (PRP) is recommended or not (Cattaneo et al, 2007; Linnemann et al,2008).

2. WHOLE BLOOD SYSTEMS

a) LOW SHEAR SYSTEMS

i. IMPEDANCE AGGREGOMETRY (WHOLE BLOOD AGGREGOMETRY)

In the whole blood impedance aggregometry (WBA) agonists are added directly into whole blood and platelet aggregation is measured through the change of impedance between electrodes, where platelets adhere and aggregate (Cardinal and Flower, 1980). Therefore no time consuming centrifugation steps are necessary. Further development of the instrument (Multiplate® -Multiplate® Platelet Function Analyzer, Figure 7) now allows simultaneous measurements through multiple channels and a computerized quality control through multiple independent sensor units in the test cells (cuvettes) (Calatzis, 2007). The test procedure is well standardized through software prompt and an automatic pipette, which provides better reproducibility and makes the device quite simple in use. As disposable cuvettes are available the assay is now less laborious. In comparison to other new tests it is rather expensive (due to disposable cuvettes) (Michelson, 2004). High sensitivity to the effects of Aspirin and Clopidogrel could be shown (von Pape et al, 2007, Sibbing et al, 2008) as well as good applicability in perioperative management for quick diagnose and therapy of platelet function disorders (Görlinger et al, 2007) with the Multiplate®. As it is an open system it is used for these clinical purposes (monitoring of platelet inhibitors, assessment of perioperative platelet function disorders or screening for platelet function defects like GT or BSS) but can also be modified for research application (http://www.Multiplate®.net).

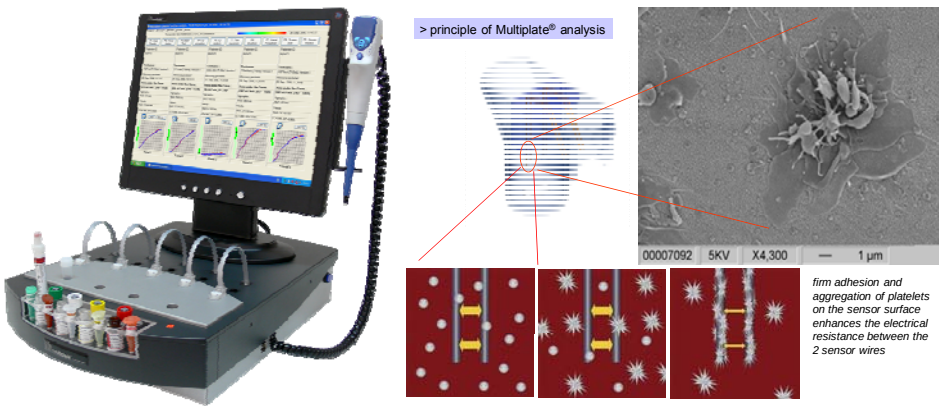


Figure 7: Multiplate® and its test principle (www.Multiplate®.net)

ii. VASP PHOSPHORYLATION FLOW CYTOMETRIC ASSAY

The vasodilatator-stimulated phosphoprotein (VASP) phosphorylation assay (VASP assay) is used to test the inhibition of the ADP-receptor P_{2Y12} and therefore is a very specific method for the evaluation of the efficacy of agents that inhibit the ADP-receptor such as Clopidogrel (Aleil et al, 2005) (Figure 8). Advantages of this test are, that it is directly dependent on the P_{2Y12} receptor and that only a small sample volume is required; on the other hand, the method is relatively cost-intensive, sample preparation and a specialized operator are needed (Michelson, 2004) and although the method protocol is well standardized differences in the calculation of PRI (platelet reactivity index) lead to variable results (Siller-Matula et al, 2009). The test has shown good results for detecting reduced platelet inhibition and therefore is useful in predicting stent thrombosis or MACEs after stenting (Bonello et al, 2007; Barragan et al, 2003). Furthermore, there is evidence that it is not sensitive to inherited P_{2Y12} deficiency because normal results were obtained (Cattaneo, 2007).

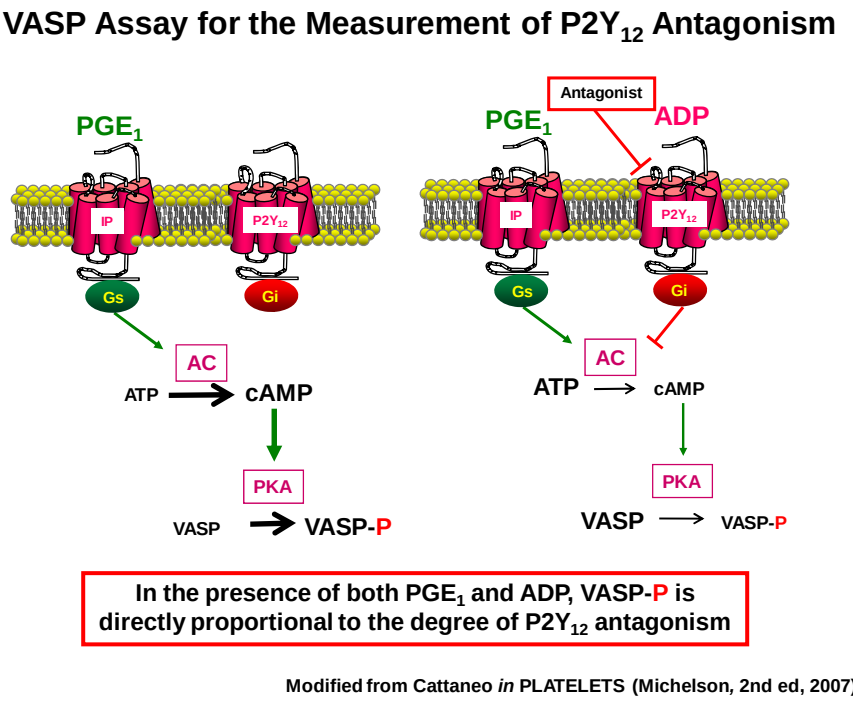


Figure 8: Schematic illustration of the VASP assay principle (modified from Cattaneo in *Platelets* (Michelson AD, 2nd ed, 2007)

b) HIGH SHEAR SYSTEMS

These devices were developed with the attempt to simulate the in vivo processes during vessel wall damage, platelet function is tested under high shear conditions (Harrison et al, 2007)

i. CONE AND PLATE(LET) ANALYZER

In 1997 Savion and Varon presented the first instrument to test whole blood platelet adhesion and aggregation on a plate coated with extracellular matrix (Varon et al, 1997) which was replaced through polystyrene plates to make it commercially available (Impact™(-R), Figure 9) (Harrison, 2005). With the cone and plate(let) analyzer (CPA) platelet adhesion and aggregation can be tested under close to physiologic conditions by inducing high shear on the polystyrene well. It is possible to test platelet function in a screening test as well as agonist induced platelet aggregation (Savion and Varon, 2006; Shenkman et al, 2008). It is a simple and rapid method where only a small sample of whole blood is required (Michelson, 2004; Harrison et al, 2007), otherwise the system is not well standardized yet and results are depending relatively strong on the operator. This method, requiring platelet activation and the presence of fibrinogen, vWF and their receptors, can be used to assess platelet function disorders (Shenkman et al, 2000) such as vWD or GT (Varon et al, 1997). There is evidence for the usefulness of the CPA for monitoring the function of transfused platelets in patients with BSS or other severe platelet disorders (Panzer et al, 2007). The Impact-R™ may also be a useful instrument for the evaluation of platelet function under platelet harvesting or storage procedures (Jilma-Stohlawetz et al, 2008). Furthermore, it is a good method for monitoring the effects of anti-platelet drugs (Shenkman et al, 2008; Spectre et al, 2005). In a relatively small study, Matetzky et al could detect poor response to Clopidogrel in a significant percentage of STEMI (ST-segment-elevation acute myocardial infarction) patients with the Impact™ (Matetzky et al, 2004).

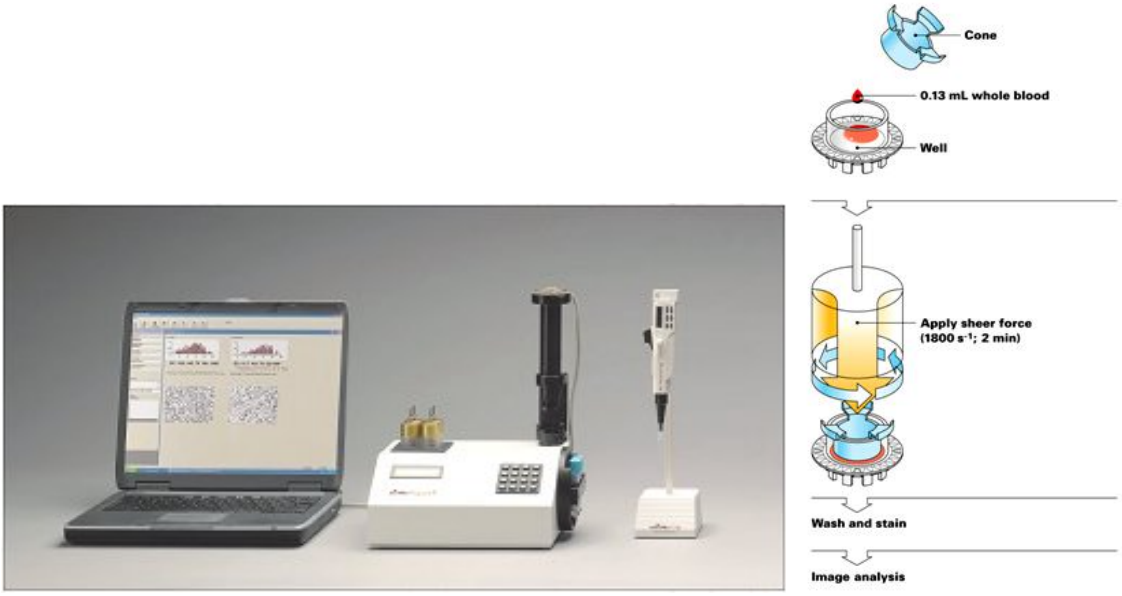


Figure 9: Impact-R™ (www.diamed.com) and test principle (www.matis-medical.com)

ii. PLATELET FUNCTION ANALYZER

With the platelet function analyzer (PFA-100®) the in-vitro-bleeding time can be measured (Figure 10). The first instrument was presented in 1985 (Kratzer and Born, 1985) and was further developed to the now available PFA-100®. The instrument simulates high shear in disposable cartridges. Whole blood is aspirated through a small aperture in a membrane that is coated with collagen and either epinephrine or ADP, platelets get activated and form a hemostatic clot. The time until occlusion of the aperture is measured. The device is easy in use, no sample preparation is needed, the assay time is short and furthermore it is characterized through good precision and reliability (Michelson, 2004; Cattaneo, 2007; Jilma, 2001). Many studies have tested the clinical applicability (Jilma, 2001). The assay has found to be sensitive to vWD and other severe platelet disorders such as GT or BSS, but not very sensitive to mild, inherited or drug induced platelet dysfunctions (Hayward et al, 2006). This can be due to the high sensitivity of the method to different variables influencing platelet aggregation, such as platelet count, platelet reactivity to collagen and plasma vWF (Cattaneo, 2004) and is therefore not a reliable assay for testing the efficacy of Aspirin and Clopidogrel (Cattaneo, 2007)

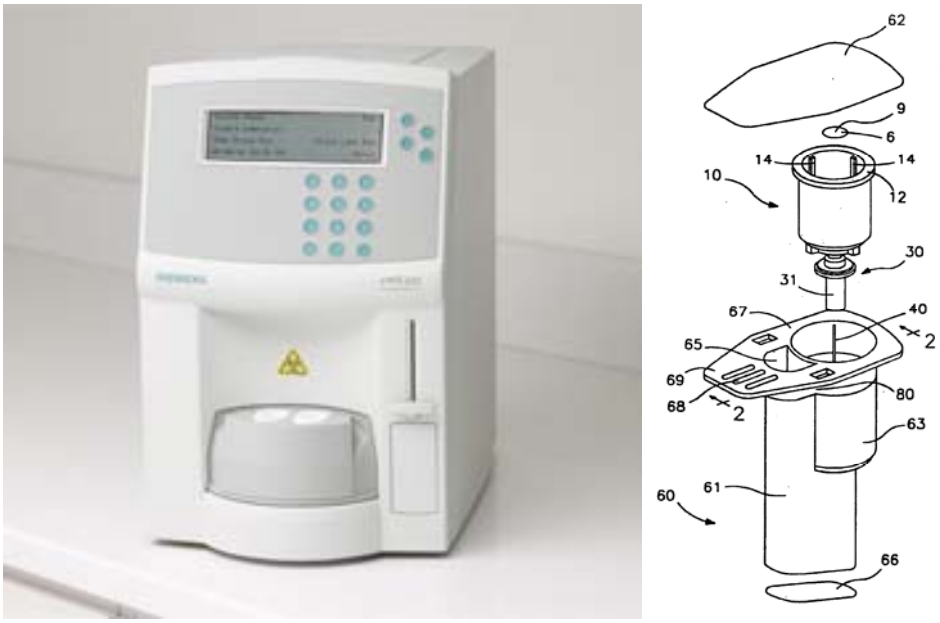


Figure 10: PFA-100® device (http://diagnostics.siemens.com) and schematic illustration of a test cartridge (http://www.freepatentsonline.com/6702987.html)

II. AIM OF THIS STUDY

There are not many studies that compare more than two methods for testing the platelet function in patients on dual anti-platelet therapy, with inherited and acquired diseases or other bleeding complications.

The different laboratory methods described above (WBA (Multiplate®), VASP phosphorylation assay, CPA (Impact™ and Impact-R™), and the platelet function analyzer (PFA-100®) were evaluated in comparison to the "gold standard" method LTA in their ability to detect resistance to Aspirin and Clopidogrel.

Furthermore the feasibility of these methods for the assessment of suspected bleeding disorders was tested.

III. MATERIALS and METHODS

1. STUDY POPULATIONS

Three groups of study populations (healthy individuals and patients) were tested. The study was approved by the Ethics Committee of the Medical University of Vienna. All participants were informed about the purpose of the study and gave informed consent.

1. Reference population

(Population 1)

39 healthy volunteers, aged 18 to 73 years, were tested to obtain normal ranges for each parameter. All subjects were asked about their smoking habits, intake of garlic, fish oil preparations, olive oil within the last 24 hours and blood group (if known) (Table 1).

Females were additionally asked about the use of hormonal contraceptives. One person had a gestagenic implant, all other positive persons reported to take oral contraception (Table 1).

The information was recorded through standardized questionnaires.

Table 1: Characteristics of reference population

REFERENCE POPULATION		
n		39
Male/Female *n*		15/24
Age *(median)*		32,5
Smoking (%)		33,4
Intake of fish oil preparations, olive oil, garlic (%)		12,8
Contraceptive (hormonal) (%)		12,9
Bloodgroup *n*	0+	10
	0-	2
	A+	16
	A-	1
	B+	4
	B-	1
	AB+	3
	AB-	0

Subjects were recruited at the Clinical Department of Blood Group Serology, Medical University of Vienna and selected according to the following criteria.

Inclusion criteria:
- Equal or greater than 18 years of age
- Apparently healthy
- Platelet count between 120 to 400 G/l

Exclusion criteria:
- Any medication, except contraceptive pills
- Pregnancy
- History of bleeding or thromboembolic complications
- Platelet count <120 G/l or > 400G/l
- Haemoglobin <10g/dl and/or haematocrit <30%

2. Patients on anti-platelet therapy

(Population 2)

32 Patients on therapy with Aspirin and Clopidogrel or Aspirin only were analyzed. Subjects, both males and females, aged between 41 and 89 years, were asked about their smoking habits, intake of garlic, fish oil preparations, olive oil within the last 24 hours and blood group (if known) (Table 2). Given information and the enacted daily dose of Aspirin and Clopidogrel respectively, were recorded through standardized questionnaires.

Table 2: Characteristics of patients on anti-platelet therapy

ANTI-PLATELET THERAPY POPULATION	
n	32
Male/Female *n*	27/5
Age *(median)*	63
Smoking (%)	6,2
Intake of fish oil preparations, olive oil, garlic (%)	3,1
Medication	Aspirin (100mg) 8 Aspirin (100 mg) + Clopidogrel (75 mg) 22 Aspirin (100 mg) + Clopidogrel (150 mg) 2
Bloodgroup *n*	0+ 8 0- 0 A+ 5 A- 1 B+ 6 B- 1 AB+ 4 AB- 0

All patients were recruited at the Department of Internal Medicine II and selected according to the following criteria.

Inclusion criteria:
- Equal or greater than 18 years of age
- Receiving Aspirin or Clopidogrel or both

Exclusion criteria:
- Abnormal Impact™ screening test
- Pregnancy
- History of bleeding complications
- Platelet count <120 G/l
- Haemoglobin <10g/dl and/or haematocrit <30%
-

3. Patients with vWF deficiency or platelet disorders

(Population 3)

This study population consisted of two persons with vWD (one was tested twice), 12 other patients with bleeding complications recruited from the Clinical Division of Haematology and Haemostaseology, Department of Internal Medicine I , and 5 patients with end stage renal failure recruited at the Division of Nephrology and Dialysis. Males and females, aged between 25 and 89, were included and asked about their smoking habits, intake of garlic, fish oil preparations, olive oil within the last 24 hours and blood group (if known). Given information was recorded through standardized questionnaires (Table 3).

Table 3: Characteristics of patients with platelet disorders

PLATELET DISORDER POPULATION		
n		20
Male/Female *n*		5/15
Age *(median)*		44,5
Smoking (%)		15
Intake of fish oil preparations, olive oil, garlic (%)		15
Bloodgroup *n*	0+	12
	0-	0
	A+	4
	A-	0
	B+	2
	B-	0
	AB+	0
	AB-	0

Additionally vWF-Antigen (vWF: AG), vWF-Multimers, vWF Ristocetin-CoFactor (vWF: RiCoF) and Fibrinogen values were recorded, if known.

Patients were selected according to the following criteria.

Inclusion criteria:
- Equal or greater than 18 years of age
- Confirmed vWD or other bleeding complications

Exclusion criteria:
- Pregnancy
- Platelet count <120 G/l
- Haemoglobin <10g/dl and/or haematocrit <30%

The blood samples of the three study populations were analyzed between the end of June 2007 and January 2008.

2. BLOOD SAMPLING

A maximum of 12 ml buffered citrated blood (3,8 % Na-citrate) and 4ml of heparin blood were obtained from each person by venipuncture using a 21 gauge needle and evacuated tubes containing sodium citrate and heparin respectively (Vacuette® Blood Collection Set and tubes, Greiner Bio-one, Kremsmünster, Austria).

Blood was drawn with minimum stasis, avoidance of foam formation and mixed gently after collection. The first few drops were discarded.

All samples were analyzed after 30 to 180 minutes after collection.

WHOLE BLOOD COUNT

A whole blood count (WBC) was performed with a Beckman/Coulter T540 Haematology Analyzer® (Beckman Coulter Inc., Fullerton, CA) for each individual, mainly to get information about platelet number, haematocrit and haemoglobin because these factors influence the sample analysis results. Additionally, white and red blood cell count and mean cell volume (MCV) were recorded.

3. SAMPLE ANALYSIS

1. LIGHT TRANSMISSION AGGREGOMETRY

The LTA method is based on the change in turbidity in PRP while platelets aggregate. This process is induced by added agonists.

For measurement a cuvette containing PRP is placed in an apparatus where a light beam passes the sample. As the agonist is added and platelets aggregate, the optical density in the sample decreases and more light passes. The increase in aggregation is recorded as a platelet aggregation curve, which provides information about the percentage of aggregation (maximum amount of

change in light transmission), slope of the aggregation (in % change of aggregation per minute) and the area under the aggregation curve within a defined period (start- and endpoint). Platelet poor plasma (PPP) is used as a reference value (100% aggregation).

a) PREPARATION OF PRP and PPP

After blood collection samples rested for at least 10 minutes before they were stored uncapped in a tilted position (45°) for 20 minutes. PRP was subsequently obtained by centrifugation of citrated blood (3,8% sodium citrate) for 10 minutes at 150g (850 rpm) and room temperature without break (Rotixa / AP centrifuge, Hettich, Tuttlingen, Germany).

PRP was removed with a pipette with a large inner diameter (to avoid activation of platelets) and PPP was produced by further centrifugation at 1200g (3000 rpm) for 5 minutes at room temperature (Rotanta / AP centrifuge, Hettich, Tuttlingen, Germany) and a following centrifugation of the obtained PPP for 5 minutes at 13.000 rpm (13.000g) (Heraeus Biofuge fresco, Kendro Laboratory Products, Hanau, Germany).

PRP platelet count was measured with the Beckman/Coulter T540 Haematology Analyzer®. Tubes containing PRP were stored uncapped until aggregation was tested.

b) AGGREGOMETRY

In this study a Chrono-Log Model 490-4D optical four channel aggregometer (Chrono-Log Corporation, Havertown, USA) was used for performing the LTA. The associated AGGRO/LINK® Software for Windows was used on the connected computer (IBM) and results were printed out on an external printer.

To analyze agonist induced platelet aggregation, 225µl of PRP were pipetted into a cuvette (with spacer on bottom and stir bar inside) and preheated to 37°C for at least 6 minutes. Subsequently 25µl of Agonist (Table 4) were added for the various tests (AA-LTA, ADP-LTA, TRAP-LTA, EPI-LTA, RISTO-LTA H and L, COL-LTA). The change in light transmission was measured for 5 minutes. If necessary, agonists were diluted with PBS to achieve the required final concentration. Startpoint (addition of agonist) was defined manually, results

were calculated by the software and printed out. Maximal aggregation was used for analysis.

Table 4: LTA-Agonists and final concentrations

LTA		
AGONIST	ALIQUOT for 0,25ml	FINAL CONCENTRATION
AA	25µl	1,6mM
ADP	25µl	11µM
TRAP-6	6,25µl (+ 18,75 PBS)	25µM
EPI	2,5µl (+22,5µl PBS)	5,5µM
RISTO H	25µl	1,6mg/ml
RISTO L	12,5µl (+12,5µl PBS)	0,8mg/ml
COL	25µl	11µg/ml

(AA=arachidonic acid, ADP= adenosine diphosphate, TRAP=thrombin receptor activating peptide, EPI=epinephrine, RISTO=ristocetin H=high, L=low, COL=collagen)

All individuals were tested with AA-LTA, ADP-LTA and TRAP-LTA. In addition, population 1 and 3 were tested with EPI-LTA and RISTO-LTA H, and population 3 also with RISTO-LTA L and COL-LTA.

2. IMPEDANCE AGGREGOMETRY

(WHOLE BLOOD AGGREGOMETRY, WBA)

To test the samples with WBA the Multiplate® (Dynabyte, Munich, Germany) device was used.

The principle of this “multiple platelet function analyzer” is based on the change of electrical resistance between two electrodes. The activated platelets adhere to and aggregate on the metal sensor wires.

The wires are located in disposable test cells. Four electrodes form two independent sensor units. Two curves are assessed because the instrument detects the impedance change of each sensor unit separately. Platelet aggregation is recorded continuously and transformed to arbitrary “aggregation units” (AU). The provided software calculates parameters; area under the aggregation curve (AUC), velocity (maximum slope of the curve), and aggregation (AU) are mean values of the parameters determined with each curve. Five channels and a fully automated pipette can be used for multiple test procedures. The apparatus is equipped with an internal Windows XP based computer system and test results are automatically saved on the system´s hard disk.

In this study 300µl of gently mixed Heparin blood were added to 300µl of prewarmed saline 0,9% in the test cell and incubated for 3 minutes at 37°C. Subsequently defined aliquots of agonists (Table 5) were added for the various tests (ASPItest (AA), ADPtest, ADPtest HS, TRAPtest, RISTOtest H, RISTOtest L). A PTFE-coated stirring magnet provided mixing of the sample. The change of the electrical resistance between the wires after addition of the agonist was recorded for 6 minutes measuring time.

Table 5: Multiplate®-Agonists and final Concentrations

MULTIPLATE®		
AGONIST	ADDITION to 0,6ml	FINAL CONCENTRATON
AA	20µl	0,5mM
ADP	20µl	6,4µM
ADP HS	20µl ADP + 20µl PGE_1	6,4µM + 9,4nM (PGE_1)
TRAP-6	20µl	32µM
RISTO H	50µl	0,77mg/ml
RISTO L	12µl	0,2mg/ml

(AA=arachidonic acid, ADP= adenosine diphosphate, ADP HS=ADP high sensitive, TRAP=thrombin receptor activating peptide, RISTO=ristocetin H=high, L=low)

Following tests were performed:

Population 1: ASPItest, ADPtest, ADPtest HS, TRAPtest, RISTOtest H.
Population 2: ASPItest, ADPtest, ADPtest HS, TRAPtest
Population 3: ASPItest, ADPtest, TRAPtest, RISTOtest H +L.

3. VASP PHOSPHORYLATION FLOW CYTOMETRIC ASSAY

The vasodilatator-stimulated phosphoprotein (VASP) phosphorylation assay (VASP assay) is used to test the inhibition of the ADP-receptor P_{2Y12} and the platelet responsiveness to Clopidogrel respectively. The phosphorylation of VASP is regulated by the cAMP (cyclic adenosine monophosphate) cascade, which is activated through PGE_1 (prostaglandin E_1) and inhibited by ADP.

In this study the VASP phosphorylation was determined using a standardized flow cytometric assay (Platelet VASP; BioCytex, Marseille, France), which is an adaption of the method of Schwarz et al (Schwarz et al, 1999). Blood samples (3,8% sodium citrate-anticoagulated whole blood) were incubated first with PGE_1 alone or with PGE_1 + ADP. A subsequent fixation was followed by permeabilization of the platelets and labelling with a primary monoclonal antibody against serine 239-phosphorylated VASP (clone 16C2) or its isotype, followed by a secondary fluorescein isothiocyanate (FITC)-conjugated polyclonal goat-antimouse antibody (Table 6). All steps were performed at room temperature, while incubation tubes were stored without light. Finally, 1000µl of diluent-reagent were added and samples were stored at 2-8° C until analysis within 120 minutes.

Table 6: VASP assay

VASP ASSAY				
	Tube 1	Tube 2	Tube 3	Incubation
Incubation with PGE1 / PGE1 + ADP	5µl WB 5µl PGE1	5µl WB 5µl PGE1 + ADP	5µl WB 5µl PGE1 + ADP	10 min RT, dark
Fixation	5µl fixative reagent	5µl fixative reagent	5µl fixative reagent	5 min RT, dark
Permeabilization & *VASP-Antibody* / Isotypic Control	5µl VASP-Ab	5µl VASP-Ab	5µl Control	5 min RT, dark
Staining with FITC-IgG & CD61-PE	5µl staining reagent	5µl staining reagent	5µl staining reagent	5 min RT, dark

(WB = whole blood)

For determination of the mean fluorescence intensity (MFI) a FACSCalibur flow cytometer (Becton Dickinson, BD, San Jose, CA) was used. The device was calibrated daily with standard BD Calibrate beads containing specific amounts of “mean equivalent soluble fluoresceine molecules” in combination with the CELLQuestPro software. The platelet population was identified by its forward and side scatter distribution, a total of 10.000 events were gated and analyzed for MFI.

Platelet reactivity was expressed as PRI calculated as shown in Figure 11. The ratio is expressed as mean percentage of platelet reactivity, inversely correlated with the Clopidogrel treatment efficiency.

$$MFIc_{(PGE1)} = MFIc_{(T1)} = MFI\ (T1) - MFI\ (T3)$$

$$MFIc_{(PGE1+ADP)} = MFIc_{(T2)} = MFI\ (T2) - MFI\ (T3)$$

$$\mathbf{PRI}_{VASP\ [\%]} = [(MFIc_{(PGE1)} - MFIc_{(PGE1+ADP)}) / MFIc_{(PGE1)}] \times 100$$

Figure 11: Platelet Reactivity Index (T1, T2, T3 = Tube 1, 2, 3)

The VASP assay was performed with all patients on therapy with Clopidogrel.

Normal samples were also analyzed in parallel as normal controls.

4. CONE AND PLATE(LET) ANALYZER (CPA)

a) Impact-R™

With the Impact-R™ device (DiaMed, Cressier, Switzerland) platelet function, effectiveness of anti-platelet therapy and other acquired and congenital abnormalities in primary haemostasis can be tested under close to physiological conditions in whole blood. Adhesion and aggregation can be tested as laminar flow with uniform shear stress over an entire plate surface and a covering rotating cone is induced. In modified tests different agonists are added to the blood samples. Therefore platelets aggregate and do not adhere to the polystyrene well as shear stress is applied (Savion and Varon , 2006).

To test the platelet function (screening test, ST), 130µl of thoroughly mixed whole blood (1 Minute on the tube rotator at 10 rpm) were placed central on a polystyrene well (plate). For the first six analyses citrated blood (3,2% sodium citrate) was used, all following tests were done with heparin blood. Special pipette tips were used to reduce preanalytical shear stress to a minimum. Immediately after placing a specially developed acrylnitril-butadien-styrene cone, together with a bell-housing, on the well, a shear rate of 820 U/min ($2050s^{-1}$) was applied for 120 seconds.

The wells were then washed thoroughly with tap water and in succession stained with May-Grünwald solution for about one minute.

Samples were analyzed under an inverted light microscope. 7 different surface areas were captured from each run and medians of these were calculated by the DiaMed Image Analysis software (Galai, Migdal Haemek, Israel).

The percentage of surface covered (SC, %) and the average size of surface bound objects (AS, μm^2) gave the required information about platelet adhesion (surface coverage) and aggregation (average size).

In healthy subjects single and aggregates of adherent platelets are aligned in the direction of the flow. The Impact-R™ ST is not sensitive to Aspirin or Clopidogrel induced platelet inhibition (Shenkman et al, 2000).

For testing agonist induced platelet aggregation (response tests), different platelet activating agonists were added to defined aliquots of the blood samples (Table 7). After gently mixing the reaction tubes and incubation for one minute on the tube rotator at 10 rpm, 130µl of the sample were placed on the well and the procedure was subsequently performed as described for the ST.

For each individual a ST was performed. In population one 1 and 2 additionally AA- and ADP-response tests were done. Like Shenkman et al. (2008) only SC values were used for testing the response to Aspirin and Clopidogrel in population 2. Furthermore a TRAP (thrombin receptor activating peptide) test was done in population 3 and in patients with vWD (population 3) a RISTO L test was added.

Table 7: Impact-R™-Agonists and final Concentrations

	IMPACT-R™		IMPACT™	
AGONIST	FINAL CONC	Aliquot for 0,4 ml	FINAL CONC	Aliquot for 0,3 ml
AA	275µM	7µl	320µM	6µl
ADP	2µM	8µl	1,38µM	3,75µl
TRAP-6	25µM	10µl	2,5µM	6µl
RISTO L	1mg/ml	25µl	0,44mg/ml	8µl

(AA=Arachidonic acid, ADP= adenosine diphosphate, TRAP=Thrombin receptor activating peptide, RISTO=Ristocetin, L=low)

b) DiaMed Impact™ Analyzer

The Impact™ (DiaMed, Cressier, Switzerland) is a fully automated version of the Impact-R™ and was designed to have a POC-device which is easy to operate and as safe as possible, so that less experienced operators as in intensive care units, emergency rooms or at the patient´s bedside, can also

obtain reliable results. In this study the practicability of this newly developed apparatus was tested.

Unlike the Impact-R™ assay, citrated blood (3,8% sodium citrate) was used. For testing the effect of the most frequently used anti-platelet drugs with the AA- and ADP-response tests DiaMed provided vials with an optimized concentration of the respective freeze-dried agonist for direct reconstitution with 300µl blood. Other agonists had to be reconstituted with distilled water as for the Impact-R™ (Table 7).

In general Impact™ screening test (regular platelet function test) and agonist response tests were conducted in the same way as in the Impact-R™ assay, with the difference that blood was pipetted automatically and all steps were prompted by the software.

After the automated staining procedure 32 different surface areas were captured and medians of surface coverage (SC, %) and average size of objects (AS, μm^2) were calculated by the image analyzer. Results were printed out on a connected printer.

A daily quality control ("self test") was performed before testing.

For each individual a ST, an AA-response test, and an ADP-response tests were performed. Like Shenkman et al. (2008) only SC values were used for testing the response to Aspirin and Clopidogrel in population 2. For population 1 and 3 additional TRAP- and ristocetin-responses were analyzed.

c) Impact-R™ wells on Impact™ (IRonI)

The Impact™ analyzes 32 images while the Impact-R™ uses 7 images.

For testing the comparability of the Impact-R™ analysis with that of the Impact™ 47 Impact-R™ wells in total (14 regular platelet function test wells of healthy individuals and 10 of patients and 14 ADP-response test wells of healthy individuals and 9 of patients on anti-platelet therapy) were analyzed on the Impact™ device.

5. PLATELET FUNCTION ANALYZER

For testing the in-vitro-bleeding time the platelet function analyzer PFA-100® (Dade Behring, Marburg, Germany) was used. The system, consisting of an apparatus and disposable test cartridges, was designed to identify inherited, acquired or induced platelet dysfunction by simulating the mechanism of primary haemostasis following a vascular injury in vitro.

Whole blood is aspirated from a sample reservoir through a capillary and the aperture of a membrane (150µm) via a vacuum pump with 40mbar. The membrane is coated with 2µg equine Type I collagen as initial matrix for platelet adhesion and two different agonists, either 10µg epinephrine bitartrate (Col/EPI test cartridge) or 50µg adenosine-5´-diphosphate (Col/ADP test cartridge). At the beginning of a test the membrane is wetted with a trigger solution (9% aqueous sodium chloride). Through high shear flow conditions (5000-6000/s) within the capillary platelets become activated and form a plug subsequently, which occludes the membrane aperture. The duration until the blood flow is stopped completely is measured and recorded as closure time (CT).

To detect platelet dysfunction induced by intrinsic platelet defects, vWD or exposure to platelet inhibiting agents the Col/EPI test cartridge would be the first choice. If an abnormal result is obtained with this cartridge the Col/ADP test is used to indicate whether this abnormal result has been caused by the effect of COX-inhibitors or medications containing COX-inhibitors.

In this study 800µl of well mixed citrated blood (3,8% Na-citrate) were pipetted into the sample reservoir opening of the test cartridge (warmed-up to room temperature for 15 minutes) after placing it in the PFA-100® carousel. After 3-8 minutes measuring time the result (CT, sec) was printed out by an integral printer.

A daily quality control (“self test”) was performed before sample testing.

Both Col/EPI-test and Col/ADP test were performed with all specimens of population 2 and 3. For population 1 PFA-100® tests were not done, because normal ranges had been established in this laboratory on the same device recently.

A list of all reagents used in this study can be found in the Appendix.

4. STATISTICS

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 15.0; Chicago, IL). Descriptive statistics were calculated in the form of median and range. For testing correlations between methods Spearman´s Rho was calculated. In case of normal distribution Pearson Correlation was used (specifically noted in Results). Furthermore a Man-Whitney-U test was calculated. Two-sided P-values < 0,05 were considered statistically significant and highly significant if P < 0,01. Additionally, specificities and sensitivities were determined based on the results from LTA and Impact-R™.

IV. RESULTS

1. NORMAL VALUES

For each parameter normal ranges were determined in 39 healthy individuals. For this purpose minimum and maximum values in the reference population were calculated after elimination of extreme values. In addition, data are presented as medians.

For the LTA normal values and medians were obtained for the AA, ADP, TRAP-6, epinephrine (EPI) and ristocetin (RISTO H) (Table 8). The table shows the maximal aggregation (max.amp.), the slope, and the area under the curve (au). PRP values were also analyzed to obtain a normal range (Table 8).

Table 8: Normal ranges for LTA

LIGHT TRANSMISSION AGGREGOMETRY			
TEST	NORMAL RANGE	Median	N
AA max.amp (%)	67 - 105	90	30
AA slope	63 - 156	118,5	30
AA au	198,8 - 415,6	362,75	30
ADP max.amp (%)	66 - 94	85	29
ADP slope	88 - 140	107	29
ADP au	290,7 - 471	361,2	29
TRAP max.amp (%)	71 - 104	89	30
TRAP slope	90 - 197	151,5	30
TRAP au	261,9 - 426,4	387,4	29
EPI max.amp (%)	69 - 93	84	29
EPI slope	48 - 99	68	28
EPI au	124,4 - 369	291,2	29
RISTO H max.amp (%)	86 - 100	93	29
RISTO H slope	79 - 199	134	29
RISTO H au	343,4 - 840	403,5	29
PRP (G/l)	185 - 411	310	31

For the WBA (Multiplate®) normal values were obtained for the following agonists: AA (ASPItest), ADP (ADPtest and ADPtest HS), TRAP (TRAPtest) and ristocetin (RISTOtest) (Table 9).

Table 9: Normal ranges for Multiplate®

IMPEDANCE AGGREGOMETRY (Multiplate®)			
TEST	NORMAL RANGE	Median	N
ASPItest (AU)	67 - 147	92	35
ADPtest (AU)	46 - 134	73	36
ADPtest HS (AU)	37 - 117	62	31
TRAPtest (AU)	74 - 156	103	34
RISTOtest H (AU)	60 - 148	93	31

CPA (Impact™ and Impact-R™) normal values and medians for screening tests (ST), response tests (AA, ADP), positive control test (TRAP) and additionally ristocetin test (RISTO L - only on Impact™) are shown in Table 10.

Table 10: Normal ranges for CPA

	IMPACT™			IMPACT-R™		
TEST	NORMAL RANGE	Median	N	NORMAL RANGE	Median	N
ST SC (%)	7,3 - 16	11	39	5,5 - 18	12	34
ST AS (µm2)	19 - 41	26	38	24 - 113	43	34
AA SC (%)	0,3 - 2,8	1,2	39	0,4 - 2,9	1	35
AA AS (µm2)	22 - 41	30	39	27 - 58	42	34
ADP SC (%)	0,5 - 3,3	1,6	37	0,3 - 2,1	1,4	32
ADP AS (µm2)	24 - 59	35	38	31 - 66	46	35
TRAP SC (%)	1,5 - 10	5,7	30	0,6 - 2	1,1	9
TRAP AS (µm2)	22 - 45	30	30	32 - 62	42	9
RISTO L SC (%)	1,3 - 4,3	2,9	27	- -	-	-
RISTO L AS (µm2)	28 - 52	32	30	- -	-	-

28 wells were manually prepared and tested on the Impact™–R (ST, ADP), and have also been analyzed on the Impact™ system (Table 11).

Table 11: Normal ranges for Impact-R™ wells analyzed by Impact™

IMPACT-R™ on IMPACT™			
TEST	NORMAL RANGE	Median	N
ST SC (%)	6,8 - 19	12,5	14
ST AS (µm2)	25 - 82	38,5	14
ADP SC (%)	1,1 - 2,3	1,7	14
ADP AS (µm2)	36 - 62	42,5	14

Normal values for PFA-100® Col/ADP CT and Col/EPI CT, and the VASP assay have recently been determined on the same devices as those used in this study and therefore no new investigations were done (Table 12).

Table 12: Normal ranges for PFA-100® and VASP assay

NORMAL RANGE	
PFA-100® COL/ADP CT (sec)	74 - 120
PFA-100® COL/EPI CT (sec)	82 - 170
VASP (%)	> 70

Normal values for vWF:AG, vWF:RiCoF and Fibrinogen have not been calculated in this study. Values obtained in earlier studies have been used (Table 13).

Table 13: Normal ranges for vWF:AG, vWF:RiCoF and Fibrinogen

NORMAL RANGE	
VWF:AG (%)	> 40
VWF:RICOF (%)	> 40
FIBRINOGEN	190 - 390

Furthermore normal ranges and medians for WBC were calculated (Table 14).

Table 14: Normal ranges for WBC

WHOLE BLOOD COUNT			
TEST	NORMAL RANGE	Median	N
PLT (G/l)	169 - 354	258	35
HB (g/dl)	11 - 17,4	14,2	31
HEMATOCRIT (%)	32,45 - 51,1	40,65	32
MCV (fl)	77,11 - 104,83	88,1	29
ERY (T/l)	3,83 - 5,96	4,66	33
LEUCO (G/l)	4,4 - 10,7	6,84	31

2. PATIENTS ON ANTI-PLATELET THERAPY

Based on the reference values for healthy individuals (Tables 8-12) cut-off values for patients on anti-platelet therapy were defined to get information about their response to Aspirin and Clopidogrel. For the LTA and WBA response was defined as values underneath the lower threshold of the reference interval. In the other methods (CPA devices (Impact™, Impact-R™), PFA-100®, VASP assay) cut-off values were defined as upper threshold of the normal range. Values above indicate response.

1. SCREENING TESTS

(REGULAR PLATELET FUNCTION TESTS)

A screening test (ST) was only applicable with the Impact™ and Impact-R™. As platelet adhesion is not impaired by Aspirin and Clopidogrel, values within the normal range can be expected. In this study, three out of 32 patients (9,4%) had values outside the normal range for SC, AS values were higher than the normal range in two patients (6,3%) on the Impact™. On the Impact-R™ SC values in three out of 31 patients (9,7%) were out of the normal range. One out of 9 (11,2%) Impact-R™-wells-analyzed-on-Impact™ (IRonI) samples showed a result which was out of the range obtained in healthy controls.

Correlations between Impact™ ST and Impact-R™ ST

(Figure 12)

Correlations between Impact™ ST and Impact-R™ ST were calculated, but no significant results could be found (SC: r=0,161; p≤0,385; AS: r=0,256; p≤0,150). Between Impact™ and IRonI ST no significant correlation was found either (SC: r=0,260; p≤0,468; AS: r=0,018; p≤0,960). Only results obtained by Impact-R™ showed expected significant and highly significant correlation with results of IRonI (SC: r=0,746; p≤0,013; AS: r=0,901; p≤0,0001). Concordant results between methods were obtained more often for AS values than for SC values. Concerning analysis with IRonI best concordance was found with Impact™ SC and Impact-R™ SC, respectively.

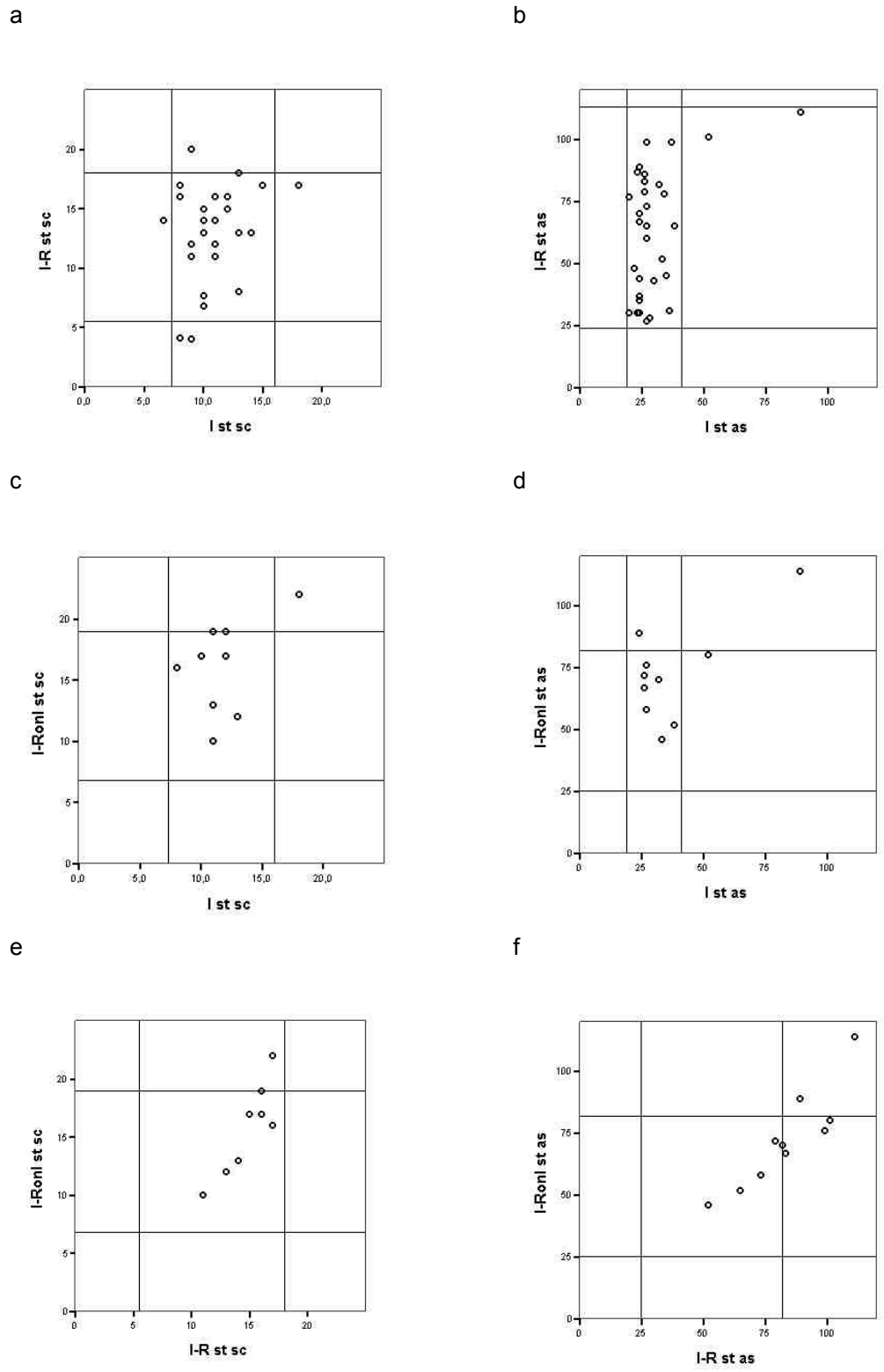


Figure 12: Relationship between Impact™, Impact-R™ , Impact-R™-analyzed-on-Impact™ (IRonI) screening tests (st)
The figure shows scatterplots with recorded vertical and horizontal reference lines (upper and lower threshold of the normal range). The central square shows concordance in screening tests between methods. Concordant results for Impact™ (I st) and Impact-R™ (I-R st) are shown in (a) for SC and in (b) for AS. (c) and (d) show concordance between Impact™ (I st) and IRonI SC and AS respectively. Concordant results between Impact-R™ (I-R st) and IRonI for SC and AS are shown in (e) and (f).

2. RESPONSE TO ASPIRIN

Cut-off values for response to Aspirin were defined as maximal aggregation < 67% for LTA, AU < 67 for the Multiplate® and SC > 2,8% for the Impact™, SC > 2,9% for the Impact-R™. PFA-100® CT > 170 seconds indicates response in this test. Additionally cut-off values for slope (< 63) and au (< 198,8) in the LTA were recorded.

In reference to the obtained normal ranges, response to Aspirin was found in 27 out of 30 (90%) in the LTA concerning maximal aggregation. On the Multiplate® (ASPItest) 28 out of 30 (93, 3%) patients were responders. On the Impact™ all patients (32) showed response for SC and on the Impact-R™ 19 out of 31 (61,3%) showed response for SC. On the PFA-100® 21 out of 30 (70%) patients showed sufficient Aspirin effect. Additionally LTA´s au (90%) and slope (63%) were considered.

Sensitivities and specificities of values obtained with all performed AA tests, based on the results from LTA (maximal aggregation), were also calculated and are shown in Table 15. Based on Impact™, Impact-R™ had a sensitivity of 61%.

Table 15: Sensitivities and specificities for AA based on LTA

LTA AA				
	MULTIPLATE® (AU)	IMPACT™ SC (%)	IMPACT-R™ SC (%)	PFA-100® (sec)
SENSITIVITY (%)	100	100	67	74
SPECIFICITY (%)	67	-	67	67

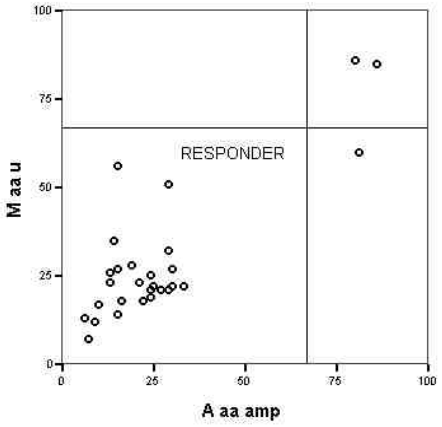
a) Correlations between AA-LTA and other methods

(Figure 13)

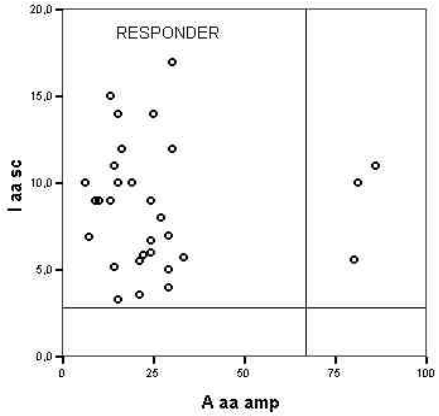
There was only a significant correlation between data obtained by LTA and Multiplate® (r=0,461; p≤0,01). All other methods did not correlate: Impact™

(r=0,-0,069; p≤0,716), Impact-R™ (r=-0,070; p≤0,715) and PFA-100® (r=-0,225; p≤0,232). Results obtained by Multiplate® showed a good concordance with those obtained by LTA. A good concordance for Impact™ SC with LTA was seen, the results with the Impact-R™ were less concordant. In PFA-100® and LTA only few patients were responders in both methods.

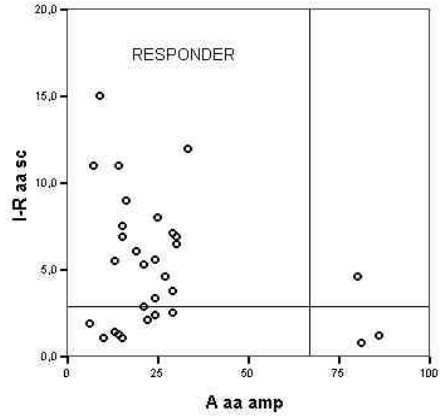
a



b



c



d

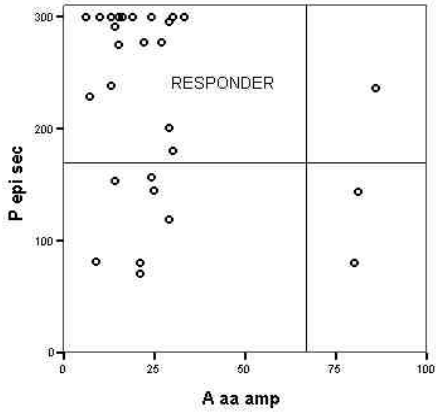


Figure 13: Relationship between AA-LTA and Multiplate®, Impact™, Impact-R™, and PFA-100®
The figure shows the relationship and concordant results in response to Aspirin (square "RESPONDER") through recorded reference lines (cut-off values) between LTA maximal aggregation (A aa amp) and Multiplate® (M aa u) (a), Impact™ (I aa sc) (b), Impact-R™ (I-R aa sc), (c), and PFA-100® (P epi sec) (d).

b) Correlations between Impact™ and Impact-R™ AA-response tests

(Figure 14)

There was no significant correlation between Impact™ and Impact-R™ AA-response test (SC: r=0,231; p≤0,212). Good concordance in response between methods according to SC was found.

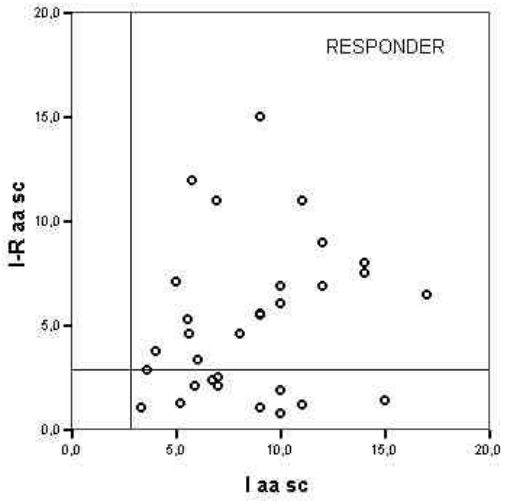


Figure 14: Relationship between Impact™ and Impact-R™ AA-response tests
The figure shows the relationship between Impact™ (I aa sc) and Impact-R™ (I-R aa sc) results and the concordance in response to Aspirin (square "RESPONDER") through recorded reference lines (cut-off values).

c) Correlations within AA-LTA

(Figure 15

Highly significant correlations were found between maximal aggregation and slope (r=0,745; p≤0,0001), respectively au (Pearson r=0,797; p≤0,0001). This was not surprising, because slope and au values were calculated based on the maximal aggregation. However, some patients, who were assigned to responders according to maximal aggregation, were no responders in respect of the slope.

a b

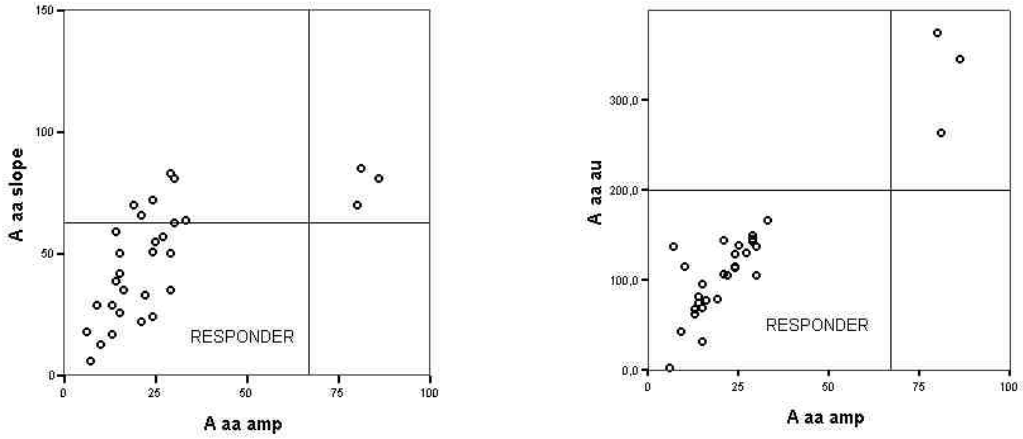


Figure 15: Relationship between AA-LTA maximal aggregation, slope and the area under the curve

The figure shows the relationship between LTA maximal aggregation (A aa amp) and slope (A aa slope) (a) and the area under the curve (A aa au) (b). The concordance in response to Aspirin (square "RESPONDER") can be seen through recorded reference lines (cut-off values).

3. RESPONSE TO CLOPIDOGREL

Cut-off values for response to Clopidogrel were defined as maximal aggregation < 66% for LTA, AU < 46 for ADPtest and AU < 37 for ADPtest HS on Multiplate® and values > 70% for VASP assay. For the Impact™ SC > 3,3%, for the Impact-R™ SC > 2,1% and for IRonI SC > 2,3% defined response. The cut-off value for Col/ADP is presumed at a CT of 120 seconds. Additionally cut-off values for slope (< 88) and au (< 290,7) in the LTA were recorded.

In reference to the obtained normal ranges, response to Clopidogrel was found in 20 out of 23 (87%) patients in the LTA concerning maximal aggregation. Values below the cut-off were found only in 7 out of 23 (30,4%) patients for ADPtest on Multiplate® but in 19 out of 23 (82,6%) for the "high sensitive" test (ADPtest HS). VASP assay had positive results in 19 out of 22 patients (86,4%).16 out of 24 patients (66,7%) for SC on the Impact™, 19 out of 23 (82,6%) on the Impact-R™ and 8 out of 9 for IRonI (88,9%) showed sufficient Clopidogrel effect. On the PFA-100® 9 out of 23 patients (39,1%) were responders. Additionally LTA slope (39,1%) and au (82,6%) were considered.

In the LTA (maximal aggregation) 5 of 6 patients (83,3%) who did not receive Clopidogrel but only Aspirin had values below the cut-off for ADP. Furthermore two of these patients had low values in LTA slope. One of these patients additionally had low values on Multiplate® and also an increased PFA-100® value. With the PFA-100® two more patients had abnormal values.

Sensitivities and specificities of values obtained with all performed ADP tests, based on the results from LTA (maximal aggregation), were also calculated and are shown in Table 16. Based on Impact™, Impact-R™ had a sensitivity of 93% and a specificity of 38%.

Table 16: Sensitivities and specificities for ADP based on LTA

LTA ADP						
	MULTIPLATE® (AU)	MULTIPLATE® HS (AU)	VASP (%)	IMPACT™ SC (%)	IMPACT-R™ SC (%)	PFA-100® (sec)
SENSITIVITY (%)	35	85	85	70	85	45
SPECIFICITY (%)	100	33	-	67	33	100

a) Correlations between ADP-LTA and other methods

(Figure 16)

Highly significant results between data obtained by LTA and Multiplate® (Pearson r=0,571; p≤0,001), Multiplate® HS (r=0,671; p≤0,0001) and Impact™ (r=-0,793; p≤0,0001), were found. With Impact-R™ (r=-0,546; p≤0,003) a significant correlation could be found. The other methods did not correlate with LTA (PFA-100®: r=-0,299; p≤0,115; VASP: r=-0,339; p≤0,123). Best concordance in response was found with Impact-R™, followed by Multiplate® ADPtest HS, VASP, Impact™, PFA-100® and Multiplate® ADPtest.

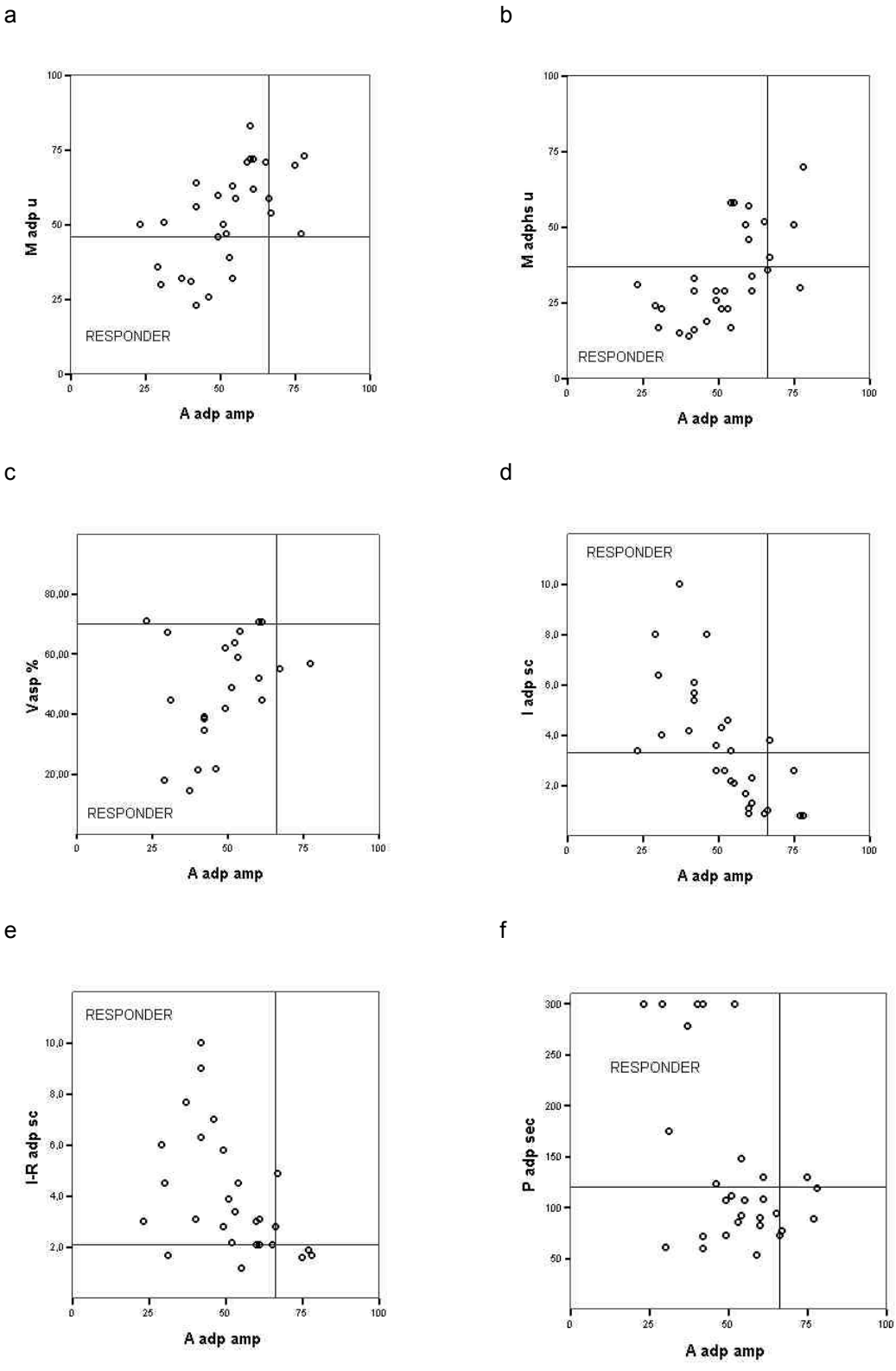


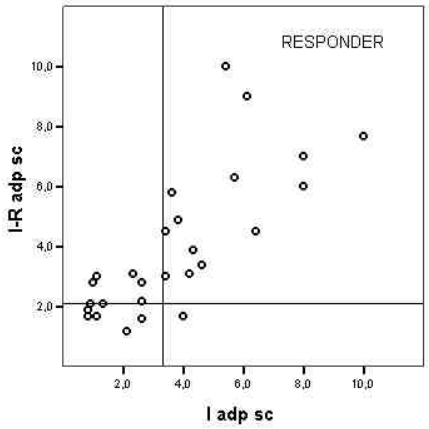
Figure 16: Relationship between ADP-LTA and Multiplate®, VASP, Impact™, Impact-R™, and PFA-100®

The figure shows the relationship and concordant results in response to Clopidogrel (square "RESPONDER") through recorded reference lines (cut-off values) between LTA maximal aggregation (A adp amp) and Multiplate® (M adp u) (a), Multiplate® hs (M adphs u) (b), VASP (c), Impact™ (I adp sc) (d), Impact-R™ (I-R adp sc) (e), and PFA-100® (P adp sec) (f).

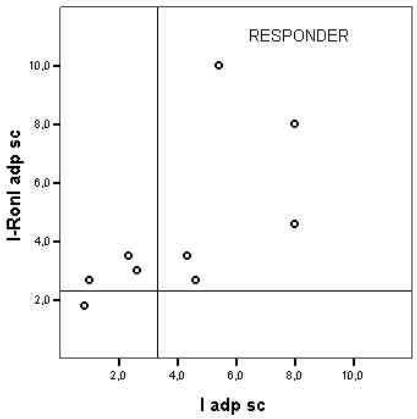
b) Correlations between Impact™ and Impact-R™ ADP-response tests (Figure 17)

Highly significant correlations between SC values obtained with Impact™ and Impact-R™ (r=0,790; p≤0,0001) and a significant correlation between those on Impact™ and IRonI (r=0,785; p≤0,012) were found. The expected highly significant correlation between Impact-R™ and IRonI was also found (r=0,907; p≤0,0001). Furthermore concordant results in ADP-response between methods were found.

a



b



c

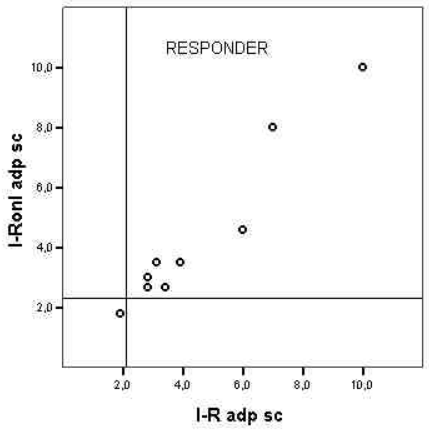


Figure.17: Relationship between Impact™ and Impact-R™ and IRonI ADP-response tests
The figure shows the relationship and the concordance in response to Clopidogrel (square "RESPONDER") through recorded reference lines (cut-off values) between Impact™ (I adp sc) and Impact-R™ (I-R adp sc) in (a), between Impact™ (I adp sc) and IRonI (I-RonI adp sc) in (b) and between Impact-R™ (I-R adp sc) and IRonI (I-RonI adp sc) in (c).

c) Correlations within ADP-LTA

(Figure 18)

Highly significant correlations were found between maximal aggregation and slope (r=0,723; p≤0,0001), respectively au (r=0,763; p≤0,0001). This was not surprising because slope and au values were calculated based on the maximal aggregation. However, according to common response, concordance between maximal aggregation values and slope was not particularly good, better results were obtained for the correlation with the area under the curve (au).

a b

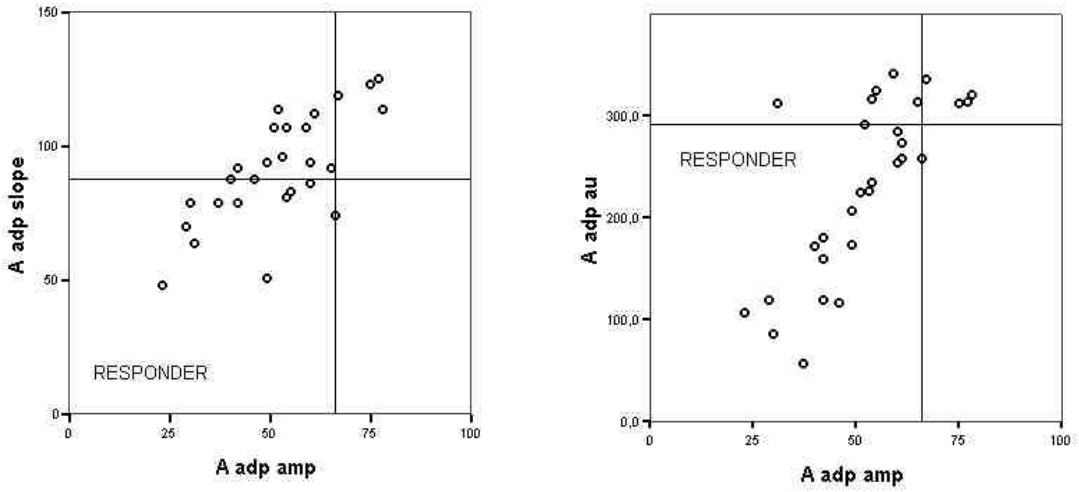


Figure 18: Relationship between ADP-LTA maximal aggregation, slope and the area under the curve

The figure shows the relationship between LTA maximal aggregation (A adp amp) and slope (A adp slope) (a) and the area under the curve (A adp au) (b). The concordance in response to Clopidogrel (square “RESPONDER”) can be seen through recorded reference lines (cut-off values).

Table 17 summarizes the response and non-response to Aspirin and Clopidogrel by the different methods.

Table 17: Response and Nonresponse to Aspirin and Clopidogrel

	DOUBLE RESPONSE	DOUBLE NON-RESPONSE	ASPIRIN RESPONSE/ CLOPIDOGREL NON-RESPONSE	ASPIRIN NON-RESPONSE/ CLOPIDOGREL RESPONSE
LTA	20	1	2	0
MULTIPLATE®	7	1	15	0
MULTIPLATE® HS	19	1	3	0
VASP	-	3	-	19
IMPACT™	16	0	8	0
IMPACT-R™	16	2	2	3
PFA-100®	8	7	7	1

4. TRAP – POSITIVE CONTROL

In all methods, where the thrombin receptor activating peptide (TRAP-6) was used, values were unexpectedly abnormal in some patients. With LTA lower values for maximal aggregation were obtained in 56,7% of patients (Z=5,779; p≤0,0001) and in 26,7% concerning both slope and au. AU on Multiplate® were lower in 27,6% and Impact-R™ SC values were higher in 16 out of 23 patients (69,6%) than in healthy controls.

a) Correlations between TRAP-LTA and other methods

(Figure 19)

A significant correlation was found only between LTA (maximal aggregation) and Impact-R™ SC (r=-0,486; p≤0,019). Multiplate® values did not correlate (r=-0,180; p≤0,350). According to concordance in response to TRAP the best result was obtained for Impact-R™ SC, followed by Multiplate®.

a b

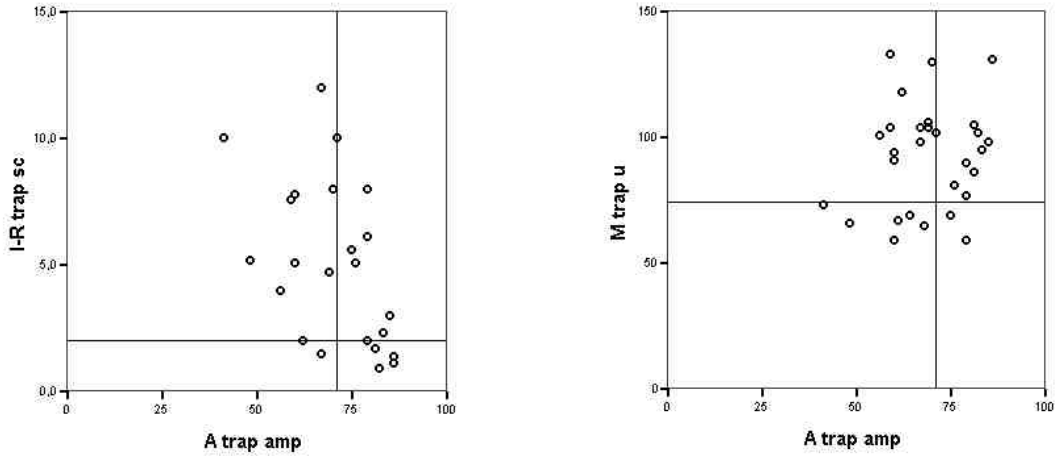


Figure 19: Relationship between TRAP-LTA and Impact™, and Multiplate®
The figure shows the relationship and concordant results through recorded reference lines (cut-off values) for the positive control between LTA maximal aggregation (A trap amp) and Impact-R™ SC (I-R trap sc) in (a) and between LTA (A trap amp) and Multiplate® (M trap u) (b).

b) Correlations within TRAP-LTA

(Figure 20

Highly significant correlations were found for both calculations, maximal aggregation with slope (Pearson r=0,731; p≤0,0001) and with au (r=0,746; p≤0,0001).

Despite good correlation, poor concordance in response for both slope and au with maximal aggregation was found, as shown in Figure 4.9.

a b

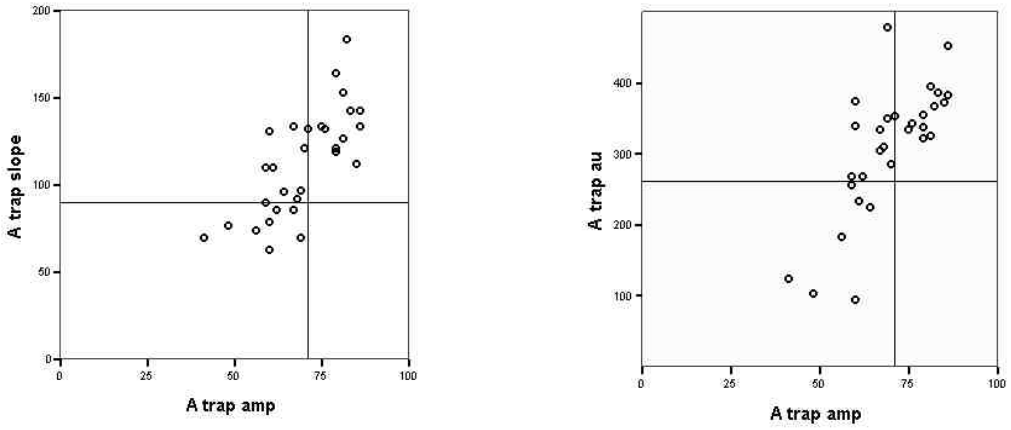


Figure 20: Relationship between TRAP-LTA maximal aggregation, slope and area under the curve

The figure shows the relationship and the concordance in positive results between LTA maximal aggregation (A trap amp) and slope (A trap slope) (a) and the area under the curve (A trap au) (b).

Examples of graphs and pictures showing results obtained with LTA (Figure III), Impact-R™ (Figure IV) and Multiplate® (Figure V) can be found in the Appendix**.**

3. PATIENTS WITH PLATELET DISORDERS

This group is described on the basis of the obtained normal values only, no statistical analyses were done. For RISTO-LTA L, Multiplate® RISTOtest L, Impact-R™ ristocetin test, and COL-LTA, tests no normal range was defined because no healthy individuals were tested.

1. BLEEDING COMPLICATIONS

In this group 12 patients with bleeding complications were tested. All patients showed normal values for vWF:AG, vWF:RiCoF and Fibrinogen.

In reference to the obtained normal ranges for platelet function tests two patients had abnormal values for the ST on Impact™ (one SC higher (9,1%), one lower (9,1%)) and on the Impact-R™ one patient (8,3%) showed an abnormally high AS value.

In the AA-LTA 3 out of 11 individuals (27,3%) had values below concerning maximal aggregation, one (9,1%) patient was found above the upper threshold. On Multiplate® (ASPItest) 3 out of 7 tested patients (42,9%) showed results below the reference range. AA-response tests on the Impact™ were abnormal in 6 patients for SC (54,5%; all above the normal range) and in 5 patients for AS (4 above (36,4%) 1 below (9,1%)). PFA-100® Col/EPI CT was prolonged in 5 patients (45,5%). Additionally AA-LTA´s slope and au values were considered abnormal (18,2%; 36,4%).

In the ADP-LTA one out of 11 (9,1%) individuals was below the normal range concerning maximal aggregation. On Multiplate® (ADPtest) all 8 tested individuals had values within the normal range. ADP-response tests on the Impact™ were abnormal only for SC in 3 patients (27,3%; all above the normal range). PFA-100® Col/ADP CT was above the normal range in 4 patients (36,4%) and below in 1 case (9,1%)., Additionally for ADP-LTA´s slope and au abnormal results were found (27,3%; 18,2%).

TRAP-LTA showed abnormal results in two patients (one higher, one lower than in healthy controls (9,1% each)) concerning maximal aggregation. The TRAPtest AU on Multiplate® were within the normal range in all tested individuals. TRAP tests on the Impact™ showed one result above and one below the normal range for SC (9,1% each). On the Impact-R™ SC values were higher in two out of 9 patients (22,2%) and below in one patient for AS (11,1%). Additionally TRAP-LTA showed abnormal results in two patients (one higher, one lower than in healthy controls (9,1% each)) for both, slope and au.

With the RISTO-LTA H three out of 11 patients (27,3%) had values below the normal range and two (18,2%) had values above the normal range concerning maximal aggregation. RISTOtest H on Multiplate® had low values in two patients (16,7%). Additionally abnormal results for RISTO-LTA H´s slope and au were found (18,2%;9,1%).

The RISTO L test on the Impact™ was higher than the normal range in 3 patients (27,3%) and below in 2 patients (18,2%) for SC. AS values were lower than in healthy controls in 2 patients (18,2%). On Multiplate® a RISTOtest L was performed in 8 patients and also RISTO-LTA L tests were done for each patient (n=11) (no reference ranges available).

With EPI-LTA 3 out of 10 individuals (30%) had values below the normal range concerning maximal aggregation, one patient was found above the upper threshold (10% each). Additionally EPI-LTA´s slope (2 below (20%), 1 above (10%)) and au (10%) values were considered abnormal. For 6 patients COL-LTA tests were performed (no reference values available).

2. END STAGE RENAL FAILURE

In this group 5 patients with end stage renal failure (ESRF) were tested. 4 patients had Fibrinogen values above the normal range.

In reference to the obtained normal ranges for platelet function tests two out of 5 patients had low SC values in the screening test (ST) on Impact™. All other ST values (Impact™ and Impact-R™) showed results within the normal range.

AA-LTA was normal in all tested patients (n=5) with all parameters. On Multiplate® (ASPItest) one out of 5 patients was found below the reference

range. AA-response tests on the Impact™ were normal in all tested patients (n=4). PFA-100® Col/EPI CT was prolonged in 4 patients.

In ADP-LTA (maximal aggregation) and with Multiplate® ADPtest all 5 tested individuals had values within the normal range. On the Impact™ ADP-response was below the normal range in one patient for AS. PFA-100® Col/ADP results were found abnormal in 4 of 5 patients (three above and one below the normal range). Additionally ADP-LTA results for slope and au were considered abnormal.

TRAP-LTA showed low results in two patients (one with maximal aggregation and one with slope) TRAPtest on Multiplate® was found below the lower threshold in one case. TRAP tests on the Impact™ showed only one result below the normal range for AS whereas on the Impact-R™ SC values in all 5 tested patients were higher than the normal range and below in 4 of 5 patients for AS. .

In the RISTO-LTA H three out of 5 patients had values below the normal range with maximal aggregation and one patient additionally showed a low result for au. RISTOtest H on Multiplate® was within the normal range in all tested individuals.

The RISTO L test on the Impact™ was lower than the normal range in one patient for SC. AS values were lower in three patients. On Multiplate® a RISTOtest L was performed only in one patient, RISTO-LTA L tests were done for all 5 patients (no reference ranges available).

With EPI-LTA three out of 5 individuals had values below the normal range concerning maximal aggregation. One patient additionally showed a low result for slope. Furthermore COL-LTA tests were performed for all 5 patients (no reference values available).

3. VON WILLEBRAND DISEASE

Two patients (A and B) with diagnosed von Willebrand Disease were analyzed. Patient B was tested twice (before and after Haemate (Haemate HS®, Aventis) infusion (2000U)).

Fibrinogen values were found above the normal range in patient A and in patient B after treatment. vWF:AG was below the normal range in patient B before treatment, vWF:RiCoF values were low in patient A and patient B before treatment.

Both patients had a very low platelet count (65; 69 and 90 after medication), patient B additionally had low hematocrit values (24,9% and 25,6%).

Therefore Impact™ and Impact-R™ ST values were very low for SC. AS values were normal except in patient B after Haemate treatment.

On both CPA devices (Impact™ and Impact-R™) no AA- and ADP-response tests were done.

AA-LTA was only done for patient B, who showed low results for maximal aggregation before treatment and for slope after treatment. With ASPItest on Multiplate® both patients showed normal results. PFA-100® Col/EPI CT was prolonged in both patients.

ADP-LTA was only done for patient B, who showed low results for all parameters before and for slope and au after treatment. With ADPtest on Multiplate® both patients showed normal results. PFA-100® Col/ADP CT was prolonged in both patients.

In the TRAP-LTA low results were obtained with all parameters in patient A, in patient B results were low concerning maximal aggregation and au before medication, after medication results were found within the normal range. With TRAPtest on Multiplate® both patients showed normal results. TRAP tests on the CPA devices were only done for patient A. On Impact™ the SC value was found below the normal range and on the Impact-R™ the AS value was found below.

In the RISTO-LTA H both patients had low values concerning maximal aggregation and au, slope was found below the normal range only in patient B after treatment. RISTOtest H on Multiplate® was only low in patient A.

The RISTO L test on the Impact™ was lower than the normal range only in patient A for SC. On Impact-R™, on Multiplate® and in the RISTO-LTA L tests were performed in both patients (no reference ranges available). EPI-LTA tests were done only with patient B. Slope was higher than the normal range before

treatment and below after treatment. For patient B COL-LTA tests were performed (no reference values available).

V. DISCUSSION

The prevalence of resistance to the anti-platelet drugs Aspirin and Clopidogrel was tested with different methodologies, the “gold standard” LTA, WBA (Multiplate®), the VASP phosphorylation assay, CPA (Impact™ and Impact-R™), and with the platelet function analyzer (PFA-100®). The investigation was performed to evaluate these tests and find the most appropriate laboratory method. Furthermore, the feasibility of these methods for the assessment of inherited, acquired and suspected bleeding disorders was tested.

For the determination of normal values minimum and maximum values were used, which have been obtained in healthy controls. However, as there is no standardization for testing platelet function by LTA results differ from one study to the next. The panel of agonists used in various laboratories is similar, but there is no consistency concerning their concentrations (Moffat et al, 2005; Jennings et al, 2008). We used agonists´ concentrations as recommended by the manufacturer. Further, we used non-parametric tests to obtain reference values, as recommended by Hayward et al (2008). Furthermore, platelets counts were not adjusted in the PRP, as recommended by Linnemann et al (2008) and Cattaneo et al (2007). The response to AA was closest to reports from others (Linnemann et al, 2008; Hayward et al, 2008). Data obtained with the other agonists like ADP, epinephrine and ristocetin, were difficult to compare. They varied widely compared to the other studies (Linnemann et al, 2008; Hayward et al, 2008), likely due to effects of the differences mentioned above but most probably caused by diverse final concentrations used for testing aggregation. In this study TRAP-6 was also used as positive control, but this agonist is not a commonly used agonist for LTA. Therefore there are no studies that report normal values.

Our normal values obtained with WBA show a wider range when compared to the ones obtained by the manufacturer (http://www.Multiplate®.net). This can be explained by the smaller sample size in our study, and also by the statistical

analyses. We report the range of data, while the reference values provided by the manufacturer are based on the 95% confidence interval.

Normal values for the CPA ST showed greater difference for SC on the Impact-R™ than on the Impact™ when compared to normal values obtained by the manufacturer. Differences in response test results between manufacturer normal values and the ones obtained in our study were minimal but showed more variability on the Impact-R™ (0,4; 0,7 difference) than on the Impact™ (0,5; 0,6 difference). This could be caused by the higher influence of the operator on variables affecting the whole procedure, from applying the sample onto the polystyrene well to finalizing the staining process or even the microscopic analysis on the Impact-R™. A discrepancy of more than ±20% was found for almost all results if Impact-R™ was compared with Impact™. Due to the use of heparin blood for the Impact-R™ we anticipated higher SC values with this instrument than with the Impact™, where citric acid anticoagulated blood was applied. Heparin may activate platelets and therefore they may better adhere to the surface of the well. However, in most of the cases SC values were higher on the Impact™. The higher operator dependency on the Impact-R™ may explain these findings. We also analyzed the Impact-R™ wells on the Impact™ device to see if the number of captured and analyzed pictures influences the results. The difference was minimal, however, for both ST and the ADP-response test.

In patients on Aspirin, most non-responders were identified by the Impact-R™, while interestingly, on the Impact™ all patients showed good response. All other methods revealed data within the range of the Impact-R™ and the Impact™.

By the LTA 90% of patients were responsive to Aspirin in our study. A similar prevalence of Aspirin resistance was reported in a variety of studies. Lordkipanidze et al (2007) studied patients with stable coronary artery disease, Harrison et al (2005) patients after ischemic attack and stroke, and Gum et al (2001) just patients from the cardiology ward. All these studies report patients with a range of Aspirin dosage from 75 to 325mg/d and the definition of response was based on a maximal aggregation <20%. Our patients were on 100mg Aspirin, and the cut-off value was based on the lower data point

obtained in healthy controls, which was 67%. Thus, the definition of cut-off values may determine the prevalence of responders.

By the use of WBA, the frequency of Aspirin resistance in our study was similar to findings of Pape et al (2007), but the authors did not disclose the dosage of the drug and they used a lower cut-off value (<30AU) than we did (<67AU). Furthermore, they used hirudin as an anticoagulant, whereas our samples contained heparin. The difference of anticoagulant may explain differences between out data and theirs.

Shenkman et al (2008) reported a similar rate of non-responders on the Impact-R™ like that obtained in our study.

If the PFA-100® is used to analyze Aspirin resistance a similar prevalence is reported in several studies (Andersen et al, 2002; Gum et al, 2001; Roller et al, 2002; Harrison et al, 2005). However, the Col/EPI CT is censored and this may explain that most studies obtain similar results.

As the LTA is considered to be the “gold standard” for testing platelet function we compared the results of the LTA with those obtained with the other methods.

Not surprisingly, a significant correlation was obtained with WBA but not with any other device. Interestingly, even this correlation was rather poor, but there was quite a good concordance for the assignment of responders. Likewise, Lordkipanidze et al (2007) reported the best correlation between LTA and WBA when comparing different methods in their study. Again, even though significant, the reported correlation was rather poor.

There were no correlations between the CPA and the LTA. Concordance and sensitivity were better with the Impact™ than Impact-R™. Shenkman et al (2008) report a high similarity between LTA and the Impact-R™ for resistance. These findings are in contrast to our data. Again, applying different statistical methods may explain this discrepancy.

There was no correlation between the LTA and PFA-100® and there was also no concordance with respect to responders or non-responders. A poor correlation or over-all agreement between these methods is reported by several studies testing patients on Aspirin only (Lordkipanidzé et al, 2007; Harrison et al, 2005; Gum et al, 2001), though varying incidence in non-response was

reported. As the PFA-100®, which is widely used for testing Aspirin resistance because of its short assay time and simplicity, is a method measuring general platelet reactivity, it cannot be expected that all dependent factors are inhibited by Aspirin. As this method is non-specific to Aspirin inhibition of COX it might be not the best methodology for detecting Aspirin resistance (Lordkipanidze et al, 2007).

Based on the values obtained in healthy controls, the highest frequency of Clopidogrel non-responders was identified by the Multiplate® ADPtest, followed by the PFA-100® and the Impact™. With the other methods fewer non-responders were found.

Erlinge et al (2008) report about twice as many non-responders with the LTA in patients with stable CAD. Other than in our study, they tested on defined days after administration of 600mg loading dose. Furthermore, LTA was performed with only 5µM ADP and a lower cut-off value was used. Paniccia et al (2007) also report higher incidence of residual platelet reactivity in high risk patients, although using a very similar concentration and cut-off value. This discrepancy may be due to a smaller and less clearly defined population in our study.

With the WBA more than twice as many responders where found with the “high sensitive” test in comparison to the “normal” ADP test, which is similar to the findings of Mueller et al (2007).They tested TI-blood samples of patients with manifest CAD on Clopidogrel therapy for at least 10 days. Platelet aggregation was found to be significantly higher in patients on concomitant treatment with Calcium Channel Blockers (CCB) (Siller-Matula et al, 2008). Unfortunately, we have no information on concomitant drugs in our cohort.

Erlinge et al (2008) found about 50% poor responders with the VASP assay in their study, even though they used a lower cut-off value. They tested platelet inhibition on defined days after administration of a loading dose and daily treatment. Based on our method of evaluation, we had only 14% of non-reponders. It has been shown, that non-responders prevail in diabetic patients (Erlinge et al, 2008). We have no information on concomitant diseases in our patients, and therefore were not able to stratify our data accordingly.

Unlike previous investigations with the CPA, we based the response on data obtained in healthy controls, who were not on anti-platelet therapy. Matetzky et al (2004), and Gremmel et al (2009) based their interpretation of response on the first quartile of data of the total patient population. Therefore, the response rate defined by our method and that used by the other investigators is not comparable.

Using the PFA-100® we would assume a low frequency of responders. It has to be considered, that the Col/ADP CT test is relatively insensitive to Clopidogrel induced platelet inhibition, however (Hayward et al, 2006).

The LTA correlated more often with the other ADP-response tests than with the Aspirin response tests.

Multiplate® ADPtest results correlated highly significant, but still poor with the LTA in our study. Similar data were obtained by Gremmel et al (2008), and also Lordkipanidze et al (2008) report comparable results between WBA and LTA, although using different concentrations of agonist for both methods. Their WBA method, however, differed from our Multiplate®. Sibbing et al (2008) used different concentrations for the LTA, but reported a better correlation with the WBA. However, their study results were obtained by combining data from before and after Clopidogrel treatment and are thus not comparable with our data. With the “high sensitive” ADPtest, the correlation was stronger, more concordance between results, and a higher sensitivity was obtained. The specificity, however, was poor based on LTA.

In contrast to Gemmel et al (2008) and Paniccia et al (2007), we found no correlation between the VASP assay and the LTA. This leads to the assumption that the size and the formation of the study population might play a role.

Both CPA devices showed a significant correlation between LTA and SC. Gremmel et al (2009) found a weaker correlation, but similar concordance. Still, we obtained a higher sensitivity but a lower specificity than these authors. As they tested under similar conditions, differences may be explainable with the smaller size of the study population or the strong user dependency with the Impact-R™. Shenkman et al (2008) found similar results as Gremmel et al (2009), although other final concentrations and cut-off values for the Impact-R™ were used.

There was no correlation between the PFA-100® and the LTA. Lordkipanidze et al (2008) report highly significant correlation, but with a similar poor correlation coefficient. The difference may be explained with the differing sample size or differing concentrations in the LTA. Paniccia et al (2007) used a similar concentration in the LTA as we did and also found no correlation but higher concordance in their patients with CAD. As mentioned above, it has to be considered that the Col/ADP test is known not to be very sensitive to detect Clopidogrel induced platelet inhibition.

According to our results, evaluating the newly developed devices in comparison to the LTA, the Multiplate® system (WBA) and the CPA devices seem to be the most adequate methods for testing the response to anti-platelet agents.

Results for ST (SC) by Impact-R™ and Impact™ were similar in cardiologic patients. We observed larger differences, however, with AA- than with ADP-response tests if the two devices were compared. Handling of the prefilled agonists may explain these findings, at least in part. Correlation calculations support these findings, as a good correlation was found between ADP-response tests, concordance of results was high, resulting in a high sensitivity for Impact-R™ results based on Impact™ data. AA-response tests showed no correlation. The microscopic analysis of Impact-R™ wells on the Impact™ showed more similarity in the ST than in the ADP-response test. Highly significant correlation and good concordance were found as expected.

Interestingly, in all methods where TRAP-6 was used in cardiologic patients, values were found abnormal in up to about 70%. According to the “Multiplate® platelet function analysis – application and interpretation – folder” (Calatzis et al) lower values were found in patients treated with anti-platelet agents than in healthy blood donors. This might indicate an influence of anti-platelet drugs on the response to TRAP, which leads to the question if it is suited as a positive control test.

Patients with suspected bleeding disorders were also tested with the different methods. Due to the small sample size only the incidence of abnormal values is discussed in comparison to values obtained in our healthy subjects.

The most obvious finding in this study group was a relatively high prevalence of abnormal results with AA in all tests. As these patients were tested for suspected bleeding tendency, these results could be an indicator that Aspirin or other COX–1 pathway inhibiting agents have been taken before blood sampling. Many people do not consider Aspirin as a mentionable medication. However, also with other agonists patients showed abnormal values, which might indicate a correlation with an actual bleeding disorder as Hayward et al (2009) report for LTA. Furthermore, this group showed the fewest abnormal values for all agonists with the WBA, which could be an indication for a relatively low sensitivity of this device to identify mild bleeding disorders. However, the PFA-100® showed abnormal values for almost half of the patients with both cartridges. Indeed, the PFA-100® is the most frequently used instrument to screen for bleeding disorders (Jennings et al, 2008). The most prevalent bleeding disorder in our population is vWD. The PFA-100® was designed to detect this inherited disorder, and it may therefore be, that some of our patients indeed suffered from mild bleedings due to vWD.

In patients with ESRF similar tendencies can be found, when comparing methods. The PFA-100®, again, shows a relatively high incidence of abnormal values, whereas with the Multiplate® fewer patients had abnormal test results. The occurring AA abnormalities as found in patients with bleeding complications were not prevalent in this group.

For both patient groups, Multiplate® followed by Impact™ revealed the closest concordance with the LTA.

Although the LTA is known to be a method which tests platelet function under relatively unphysiological conditions, it is suitable for the diagnosis of inherited platelet function disorders, because it shows characteristic patterns (Budde, 2002). Comparing the traditional method with the new tests in our vWD patients, no clear tendency for the closest similarity could be found, also because of the small sample size. With both CPA devices low SC values, but no abnormal AS

values were obtained in any of the three samples, which is concordant with findings of Varon and Savion (2006) in patients with vWD.

In conclusion, the results of our study were similar to earlier findings, especially for Aspirin resistance. Evaluating the different newly developed methods, the impedance aggregometry on the Multiplate® device and the CPA are considered to be the most adequate methods for testing anti-platelet drug response. However, the Multiplate® is maybe not sensitive enough for the assessment of mild bleeding disorders. Further investigations with a higher quantity of patients are required because the small sample size of our study makes it difficult to draw definitive conclusions.

VI. REFERENCES

- Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. (2005) Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of Clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost*; 3: 85-92
- Andersen K, Hurlen M, Arnesen H, Seljeflot I. (2002) Aspirin non-responsiveness as measured by PFA-100 in patients with coronary artery disease. *Thromb Res;* 108: 37-42
- Ando M, Iwata A, Ozeki Y, Tsuchiya K, Akiba T, Nihei H. (2002) Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients. *Kidney Int*; 62: 1757 – 1763
- Andrews RK, Lopez JA, Berndt MC. (1997) Molecular mechanisms of platelet adhesion and activation. *Int J Biochem Cell Biol*; 29: 91-105
- Antithrombotic Trialists' Collaboration. (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*;324:71-86
- Barragan P, Bouvier JL, Roquebert PO, Macaluso G, Commeau P, Comet B, Lafont A, Camoin L, Walter U, Eigenthaler M. (2003) Resistance to thienopyridines: clinical detection of coronary stent thrombosis by monitoring of vasodilator-stimulated phosphoprotein phosphorylation. *Catheter Cardiovasc Interv*; 59: 295-302
- Bithell TC. (1993) The physiology of primary hemostasis. In: *Wintrobe´s Clinical Hematology*. 9th edn., Lee GR, Bithell TC, Foerster J, Athens JW, lukens Jn, eds., Philadelphia: Lea & Febinger
- Bonello L, Paganelli F, Arpin-Bornet M, Auquier P, Sampol J, Dignat-George F, Barragan P, Camoin-Jau L. (2007) Vasodilator-stimulated phosphoprotein phosphorylation analysis prior to percutaneous coronary intervention for exclusion of postprocedural major adverse cardiovascular events. *J Thromb Haemost;* 5: 1630-1636
- Budde U (2002) Diagnose von Funktionsstörungen der Thrombozyten mit Hilfe der Aggregometrie. *J Lab Med* 26: 564 – 571

- Calatzis A (2007) Vollblutverfahren zur Erfassung der primären Hämostase. *J Lab Med* 31(6): 239 - 247
- Calatzis A, Loreth R, Spannagl M. Multiplate platelet function analysis – application and interpretation
- Campbell SL, Steinhubl SR (2005) Variability in response to Aspirin: do we understand the clinical relevance? *J Thromb Haemost* 3: 665 – 669
- CAPRIE Steering Committee (1996) A randomised, blinded, trial of Clopidogrel versus Aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 348: 1329 – 1339
- Cardinal DC, Flower RJ. (1980) The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J Pharmacol Methods*; 3: 135-158
- Cattaneo M (2004) Aspirin and Clopidogrel: efficacy, safety and the issue of drug resistance. *Arterioscler Thromb Vasc Biol* 24: 1980 – 1987
- Cattaneo M, Gachet C. (1999) ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol*; 19: 2281-2285
- Cattaneo M, Lecchi A, Zighetti ML, Lussana F. (2007) Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica*; 92: 694-697
- Cattaneo M. (2007) Resistance to antiplatelet drugs: molecular mechanisms and laboratory detection. *J Thromb Haemost*; 1:230-237
- Celi, A., Merrill – Skoloff, G., Gross, P. et al. (2003) Thrombus formation: direct real – time observation and digital analysis of thrombus assembly in a living mouse by confocal and widefield intravital microscopy. *J Thromb Haemost*; 1:60 – 68
- DiChiara J, Bliden KP, Tantry US, Hamed MS, Antonino MJ, Suarez TA, Bailon O, Singla A, Gurbel PA. (2007) The effect of Aspirin dosing on platelet function in diabetic and nondiabetic patients: an analysis from the Aspirin-induced platelet effect (ASPECT) study. *Diabetes*; 56: 3014-3019
- Eikenboom JC, Tjernberg P, Van Marion V, Heering KJ (2007) Acquired von Willebrand syndrome: diagnostic problems and therapeutic options. *Am J Hemato;* 82:55-8

- Erlinge D, Varenhorst C, Braun OO, James S, Winters KJ, Jakubowski JA, Brandt JT, Sugidachi A, Siegbahn A, Wallentin L. (2008) Patients with poor responsiveness to thienopyridine treatment or with diabetes have lower levels of circulating active metabolite, but their platelets respond normally to active metabolite added ex vivo. *J Am Coll Cardiol*; 52: 1968-1977
- Federici AB, Budde U, Rand JH. (2004) Acquired von Willebrand syndrome 2004: international registry. *Haemostaseologie*; 24: 50 - 55
- Federici AB, Rand JH, Bucciarelli P, Budde U, van Genderen PJJ, Mohri H, Meyer D, Rodeghiero F, Sadler JE. (2000) Acquired von Willebrand syndrome: data from an international registry. On behalf of the Subcommitee on von Willebrand factor. *Thromb Haemost*; 84: 345–349
- Fitzgerald DJ, Maree A. (2007) Aspirin and Clopidogrel resistance. *Hematology Am Soc Hematol Educ Program*; 2007: 114-120
- Frere C, Cuisset T, Quilici J, Camoin L, Carvajal J, Morange PE, Lambert M, Juhan-Vague I, Bonnet JL, Alessi MC. (2007) ADP-induced platelet aggregation and platelet reactivity index VASP are good predictive markers for clinical outcomes in non-ST elevation acute coronary syndrome. *Thromb Haemost*; 98: 838-843
- Gachet C, Hechler B, Leon C, Vial C, Leray C, Ohlmann P, Cazenave JP. (1997) Activation of ADP receptors and platelet function. *Thrombosis and Haemostasis*; 78: 271-275
- Gawaz M. (2001) *Blood Platelets*. Stuttgart: Thieme Verlag
- Gawaz MP, Dobos G, Spath M, Schollmeyer P, Gurland HJ, Mujais SK. (1994) Impaired function of platelet membrane glycoprotein IIb-IIIa in end-stage renal disease. *J Am Soc Nephrol*; 5: 36 – 46
- George JN, Caen JP, Nurden AT. (1990) Glanzmann´s thrombasthenia: the spectrum of clinical disease. *Blood*; 75: 1383 – 1395
- George JN. (2000) Platelets. *Lancet*; 355: 1531 – 1539
- Gill JC, Endres-Brooks J, Bauer PJ, Marks Jr WJ, Montgomery RR. (1987) The effect of AB0 blood group on the diagnosis of von Willebrand disease. *Blood*; 69: 1691 – 1695
- Görlinger K, Jambor C, Hanke AA, Dirkmann D, Adamzik M, Hartmann M, Rahe-Meyer N (2007) Perioperative Coagulation Management and

Control of Platelet Transfusion by Point-of-Care Platelet Function Analysis. *Transfus Med Hemother* 34: 396 – 411

- Gremmel T, Steiner S, Seidinger D, Koppensteiner R, Panzer S, Kopp CW. (2009) Comparison of methods to evaluate Clopidogrel-mediated platelet inhibition after percutaneous intervention with stent implantation. *Thromb Haemost;* 101: 333-339
- Grossmann R, Sokolova O, Schnurr A, Bonz A, Porsche C, Obergfell A, Lengenfelder B, Walter U, Eigenthaler M. (2004) Variable extent of Clopidogrel responsiveness in patients after coronary stenting. *Thromb Haemost*; 92: 1201-1206
- Guidelines on platelet function testing. The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force. (1988) [No authors listed] *J Clin Pathol;* 41: 1322-1330
- Gum PA, Kottke-Marchant K, Poggio ED, Gurm H, Welsh PA, Brooks L, Sapp SK, Topol EJ. (2001) Profile and prevalence of Aspirin resistance in patients with cardiovascular disease. *Am J Cardiol;* 88: 230-235
- Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. (2003) A prospective, blinded determination of the natural history of Aspirin resistance among stable patients with cardiovascular disease. *J Am Coll Cardiol*; 41: 961-965
- Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. (2003) Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation;* 107: 2908-2913
- Harrison P, Segal H, Blasbery K, Furtado C, Silver L, Rothwell PM. (2005) Screening for Aspirin responsiveness after transient ischemic attack and stroke: comparison of 2 point-of-care platelet function tests with optical aggregometry. *Stroke;* 36: 1001-1005
- Harrison P. (2005) Platelet function analysis. *Blood Reviews*; 19: 111 – 123
- Harrison P. Frelinger III A.L., Furman M.I., Michelson A.D. (2007) Measuring antiplatelet drug effects in the laboratory. *Thrombosis Research*; 120 (3): 323 – 336
- Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK; The Platelet Physiology Subcommittee of the Scientific and Standardization

Committee of the International Society on Thrombosis and Haemostasis. (2006) Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. *J Thromb Haemost*; 4: 312-319

- Hayward CP, Moffat KA, Pai M, Liu Y, Seecharan J, McKay H, Webert KE, Cook RJ, Heddle NM. (2008) An evaluation of methods for determining reference intervals for light transmission platelet aggregation tests on samples with normal or reduced platelet counts. *Thromb Haemost;* 100: 134-145
- Hayward CP, Pai M, Liu Y, Moffat KA, Seecharan J, Webert KE, Cook RJ, Heddle NM. (2009) Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. *J Thromb Haemost;* Article in press
- Himmelfarb J, Holbrook D, McMonagle E, Ault K (1997) Increased reticulated platelets in dialysis patients. *Kidney Int* 51: 834–839
- Himmelfarb J, Nelson S, McMongale E, Holbrook D, Benoit SE, Michelson AD, Ault K. Elevated plasma glycocalicin levels and decreased ristocetin-induced platelet agglutination in hemodialysis patients. *Am J Kidney Dis* 1998; 32: 132 – 138
- Hörl WH. (2006) Thrombozytopathie und Blutungskomplikationen bei Urämie. *Wien Klin Wochenschr*; 118: 134 – 150
- Hübl W, Assadian A, Lax J, Meixner U, Fang IF, Hagmüller G, Panzer S, Bayer PM (2007) Assessing Aspirin-induced attenuation of platelet reactivity by flow cytometry. *Thromb Res* 212: 135 - 143
- HUMATE-P, CSL Behring, 2009; http://www.humate-p.com, DOR: 14.12.2008
- Jennings I, Woods TA, Kitchen S, Walker ID. (2008) Platelet function testing: practice among UK National External Quality Assessment Scheme for Blood Coagulation participants, 2006. *J Clin Pathol*; 61: 950-954
- Jilma B. (2001) Platelet function analyzer (PFA-100): a tool to quantify congenital or acquired platelet dysfunction. *J Lab Clin Med;* 138: 152-163

- Jilma-Stohlawetz P, Horvath M, Eichelberger B, Koren D, Jilma B, Panzer S. (2008) Platelet function under high-shear conditions from platelet concentrates. *Transfusion;* 48: 129-135
- Kaufman JS, O´Connor TZ, Zhang JH, Cronin RE, Fiore LD, Ganz MB, Goldfarb DS, Peduzzi PN. (2003) Veterans Affairs Cooperative Study Group on Hemodialysis Access Graft Thrombosis. *J Am Soc Nephrol*; 14: 2313 – 2321
- Keeney S, Cumming AM. (2001) The molecular biology of von Willebrand disease. *Clin. Lab. Haem.*; 23: 209 – 230
- Kefalides NA. (1987) Biochemical aspects of the vessel wall. In: *Hemostasis and Thrombosis: Basic principles and clinical practice.* 2nd edn., Colman RW, Hirsch J, Marder VJ, Salzman EW, eds., Philadelphia: JB Lippincott Co
- Kooistra MP, van Es A, Marx JJ, Hertsig ML, Struyvenberg A. (1994) Low-dose Aspirin does not prevent thrombovascular accidents in low-risk haemodialysis patients during treatment with recombinant human erythropoietin. *Nephrol Dial Transplant*; 9: 1115 – 1120
- Kratzer MA, Born GV. (1985) Simulation of primary haemostasis in vitro. *Haemostasis;* 15: 357-362
- Kumar S, Pruthi RK, Nichols WL (2003) Acquired von Willebrand's syndrome: a single institution experience. *Am J Hematol;* 72: 243-247
- Linnemann B, Schwonberg J, Mani H, Prochnow S, Lindhoff-Last E. (2008) Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: an adjustment for platelet count is not necessary. *J Thromb Haemost*; 6: 677-683
- Lordkipanidzé M, Pharand C, Nguyen TA, Schampaert E, Palisaitis DA, Diodati JG. (2008) Comparison of four tests to assess inhibition of platelet function by Clopidogrel in stable coronary artery disease patients. Eur Heart J; 29: 2877-2885
- Lordkipanidzé M, Pharand C, Schampaert E, Turgeon J, Palisaitis DA, Diodati JG. (2007) A comparison of six major platelet function tests to determine the prevalence of Aspirin resistance in patients with stable coronary artery disease. Eur Heart J; 28: 1702-1708

- Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, Novikov I, Pres H, Savion N, Varon D, Hod H. (2004) Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation;* 109: 3171-3175
- Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H, Zhao F, Chrolavicius S, Copland I, Fox KA; Clopidogrel in unstable angina to prevent recurrent events trial (CURE) Investigators (2001) Effects of pretreatment with Clopidogrel and Aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet* 358: 527 - 533
- Michelson AD, Cattaneo M, Eikelboom JW, Gurbel P, Kottke-Marchant K, Kunicki TJ, Pulcinelli FM, Cerletti C, Rao AK; Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis; Working Group on Aspirin Resistance. (2005) Aspirin resistance: position paper of the Working Group on Aspirin Resistance. *J Thromb Haemost*; 3:1309-1311
- Michelson AD, Frelinger AL 3rd, Furman MI. (2006) Current options in platelet function testing. *Am J Cardiol;* 98: 4-10
- Michelson AD. (2004) Platelet function testing in cardiovascular diseases. *Circulation;* 110: 489-93
- Moffat KA, Ledford-Kraemer MR, Nichols WL, Hayward CP; North American Specialized Coagulation Laboratory Association. (2005) Variability in clinical laboratory practice in testing for disorders of platelet function: results of two surveys of the North American Specialized Coagulation Laboratory Association. *Thromb Haemost;* 93: 549-553
- Mohri H. (2003) Acquired von Willebrand syndrome: its pathophysiology, laboratory features and management. *J Thrombosis Thrombolysis*; 15: 41 – 149
- Mueller T, Dieplinger B, Poelz W, Calatzis A, Haltmayer M. (2007) Utility of whole blood impedance aggregometry for the assessment of Clopidogrel action using the novel Multiplate analyzer--comparison with two flow cytometric methods. *Thromb Res;* 121: 249-258

- Multiplate comprehensive platelet diagnostics, Dynabyte Informationssysteme GmbH, Munich, Germany, 2009; http:// www.Multiplate.net, DOR: 07.03.2009
- Nurden A. (1994) Human platelet glycoproteins. In: *Haemostasis and Thrombosis.* 3rd edn., Bloom A, Forbes CD, eds., New York: Churchill Livingstone; pp 259-285
- Nurden AT, Nurden P. (2007) Inherited thrombocytopenias. *Haematologica* 92:1158 -1164
- Nurden P, Chretien F, Poujol C, Winckler J, Borel-Derlon A, Nurden AT. (2000) Platelet ultrastuctural abnormalities in three patients with type 2B von Willebrand disease. *Brit J Haematol*; 110: 704 – 714
- Nurden P, Nurden AT. (2008) Congenital disorders associated with platelet dysfunctions. *Thromb Haemost*; 99: 253 – 263
- Paniccia R, Antonucci E, Gori AM, Marcucci R, Giglioli C, Antoniucci D, Gensini GF, Abbate R, Prisco D. (2007) Different methodologies for evaluating the effect of Clopidogrel on platelet function in high-risk coronary artery disease patients. *J Thromb Haemost;* 5: 1839-1847
- Panzer S, Eichelberger B, Koren D, Kaufmann K, Male C. (2007) Monitoring survival and function of transfused platelets in Bernard-Soulier syndrome by flow cytometry and a cone and plate(let) analyzer (Impact-R™). *Transfusion;* 47: 103-106
- Patel D, Väänänen H, Jirouskova M, Hoffmann T, Bodian C, Coller BS. (2003) Dynamics of GP IIb/IIIa – mediated platelet – platelet interactions in platelet adhesion / thrombus formation on collagen in vitro as revealed by videomicroscopy. *Blood*; 101: 929 – 936
- Patrono C (2003) Aspirin resistance: definition, mechanisms, and clinical read-outs. *J Thromb Haemost* 1: 1710 – 1713
- Patrono C, Coller B, FitzGerald GA, Hirsh J, Roth G. (2004) Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest*; 126: 234S-264
- Platelet Perspectives®, Siemens Healthcare Diagnostics Inc. (2007); http://www.plateletperspectives.com, DOR:14.12.2008

- Rand, L. M., Leung, R. and Packham, M. A. (2003) Platelet function assays. *Transfusion and Apheresis Science*; 28: 307 – 317
- Remuzzi G, Benigni A, dodesini P, Schieppati A, Livio M, de Gaetano G, Day SS, Smith WL, Pinca E, Patrignani P, Patrono C. (1983) Reduced platelet thromboxane formation in uremia. *J Clin Invest*; 71: 762 – 768
- Roller RE, Dorr A, Ulrich S, Pilger E. (2002) Effect of Aspirin treatment in patients with peripheral arterial disease monitored with the platelet function analyzer PFA-100. *Blood Coagul Fibrinolysis;* 13: 277-281
- Roth GJ, Majerus PW. (1975) The mechanism of the effect of Aspirin on human platelets. I. Acetylation of a particulate fraction protein. *J Clin Invest*; 56: 624-632
- Ruf A, Frojmovic MM, Pateschke H. (1997) Platelet aggregation. In: *Platelets and their factors. Handbook for experimental pharmacology.* von Bruchhausen F, Walter U, eds., Heidelberg: Springer Verlag; pp 83-94
- Ruggeri ZM (2002) Platelets in atherothrombosis. *Nat Med* 8: 1227 – 1234
- Ruggeri ZM, Zimmerman TS. (1987) Von Willebrand Factor and von Willebrand disease. *Blood* 70: 895 – 904
- Sadler, JE (1994) A revised classification of von Willebrand disease. *Thrombosis and Haemostasis*; 71:520-525.
- Savion N, Varon D. (2006) Impact™ - The Cone and Plate(let) Analyzer: testing Platelet Function and Anti-Platelet Drug Response. *Pathophysiol Haemos Thromb*; 35: 83-88
- Schwarz UR, Geiger J, Walter U, Eigenthaler M. (1999) Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets – definition and detection of ticlopidine/Clopidogrel effects. *Thromb Haemost*; 82: 1145-1152
- Sharis PJ, Cannon CP, Loscalzo J. (1998) The antiplatelet effects of ticlopidine and Clopidogrel. *Ann Intern Med*; 129: 394-405
- Shenkman B, Einav Y, Salomon O, Varon D, Savion N. (2008) Testing agonist-induced platelet aggregation by the Impact-R™ [Cone and plate(let) analyzer (CPA)]. *Platelets;* 19: 440-446

- Shenkman B, Savion N, Dardik R, Tamarin I, Varon D. (2000) Testing of platelet deposition on polystyrene surface under flow conditions by the cone and plate(let) analyzer: role of platelet activation, fibrinogen and von Willebrand factor. *Thromb Res*; 99: 353-61
- Shenkman B., Einav Y., Salomon O., Varon D., Savion N. (2008) Testing agonist-induced platelet aggregation by the Impact-R™ [Cone and plate(let) analyzer (CPA)]. *Platelets*; 19 (6): 440 - 446
- Shields DC, Fitzgerald AP, O´Neill PA, Muckian C, Kenny D, Moran B, Cannon CP (2002) The contribution of genetic factors to thrombotic and bleeding outcomes in coronary patients randomised to IIb/IIIa antagonists. *Pharmacogenomics J* 2: 182–190
- Sibbing D, Braun S, Jawansky S, Vogt W, Mehilli J, Schömig A, Kastrati A, von Beckerath N. (2008) Assessment of ADP-induced platelet aggregation with light transmission aggregometry and multiple electrode platelet aggregometry before and after Clopidogrel treatment. *Thromb Haemost;* 99: 121-126
- Siller-Matula JM, Haberl K, Prillinger K, Panzer S, Lang I, Jilma B. (2009) The effect of antiplatelet drugs Clopidogrel and Aspirin is less immediately after stent implantation. *Thromb Res;* Article in press
- Siller-Matula JM, Lang I, Christ G, Jilma B. (2008) Calcium-channel blockers reduce the antiplatelet effect of Clopidogrel. *J Am Coll Cardiol;* 52: 1557-1563
- Sirolli V, Strizzi L, Di Stante S, Robuffo I, Procopio A, Bonomini M. (2001) Platelet activation and platelet erythrocyte aggregates in end-stage renal disease patients on hemodialysis. *Thromb Haemost*; 86: 834 – 839
- Sixma JJ. (1994) Interaction of blood platelets with the wessel wall. In: *Haemostasis and Thrombosis.* 3rd edn., Bloom A, Forbes CD, eds., New York: Churchill Livingstone,; pp 259-285
- Sloand EM, Sloand JA, Prodouz K, Klein HG, Yu MW, Harvath L, Fricke W. (1991) Reduction of platelet glycoprotein Ib in uraemia. *Br J Haematol*; 77: 375 – 381
- Spectre G, Brill A, Gural A, Shenkman B, Touretsky N, Mosseri E, Savion N, Varon D. (2005) A new point-of-care method for monitoring anti-

platelet therapy: application of the cone and plate(let) analyzer. *Platelets;* 16: 293-299

- Steinhubl SR, Berger PB, Mann JT 3rd, Fry ET, DeLago A, Wilmer C, Topol EJ; CREDO Investigators (2002) Clopidogrel for the reduction of events during observation early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. *JAMA* 288: 2411 – 2420
- Thews G, Mutschler E, Vaupel P. (1999) *Anatomie Physiologie Pathophysiologie des Menschen.* 5thed. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH
- Varon D, Dardik R, Shenkman B, Kotev-Emeth S, Farzame N, Tamarin I, Savion N. (1997) A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. *Thromb Res*; 85: 283-294
- Varon D, Savion N (2006) Cone and Plate(let) Analyzer. In: Michelson AD (ed.). *Platelets.* Academic press, Amsterdam. pp 535 – 544
- Velik-Salchner C, Eschertzhuber S, Streif W, Hangler H, Budde U, Fries D. (2008) Acquired von Willebrand syndrome in cardiac patients. *J Cardiothorac Vasc Anesth*; 22:719-724
- von Pape KW, Dzijan-Horn M, Bohner J, Spannagl M, Weisser H, Calatzis A (2007) Vollblutaggregometrie zur Kontrolle der Wirksamkeit von Azetylsalizylsäure bei Patienten mit koronarer Herzkrankheit - Vergleich von PFA-100® und Multiplate [Control of Aspirin effect in chronic cardiovascular patients using two whole blood platelet function assays. PFA-100 and Multiplate]. *Hamostaseologie;* 27: 155-160
- Walkowiak B, Pawlowska Z, Michalak E, Cierniewski CS. (1994) Expression of fibrinogen receptors on platelets of uremic patients is correlated with the content of GPIIb and plasma level of creatinine. *Thromb Haemost*; 71: 164 – 168
- Wong S, Appleberg M, Ward CM, Lewis DR (2004) Aspirin resistance in cardiovascular disease: a review. *Eur J Vasc Endovasc Surg* 27: 456 – 465

- Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. (2005) Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood;* 106: 2723-2729
- Zwaginga JJ, Ijsseldijk Mj, Beeser-Visser N, de Groot PG, Vos J, Sixma JJ. (1990) High von Willebrand factor concentration compensates a relative adhesion defect in uremic blood. *Blood*; 75: 1498 – 1508

- „*Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“*

VII. APPENDIX

1. REAGENTS

Test/ Instrument	Reagent	Company
Impact™	Impact™ Test Kit	Diamed; www.diamed.com
	AA-Response Test	Diamed; www.diamed.com
	ADP-Response Test	Diamed; www.diamed.com
	DiaRistin	Diamed; www.diamed.com
	TRAP-6	Bachem;www.bachem.com
Impact-R™	Impact-R™ Test Kit	Diamed; www.diamed.com
	DiaChidon	Diamed; www.diamed.com
	DiaAdin	Diamed; www.diamed.com
	DiaRistin	Diamed; www.diamed.com
	TRAP-6	Bachem;www.bachem.com
PFA-100®	Collagen/Epinephrine (Col/EPI) Test Cartridge	Dade Behring; www.dadebehring.com
	Collagen/ADP (Col/ADP) Test Cartridge	Dade Behring; www.dadebehring.com
	Trigger Solution	Dade Behring; www.dadebehring.com
LTA	DiaChidon	Diamed; www.diamed.com
	DiaAdin	Diamed; www.diamed.com
	DiaNephrin	Diamed; www.diamed.com
	DiaRistin	Diamed; www.diamed.com
	DiaColgen	Diamed; www.diamed.com

	TRAP-6	Bachem;www.bachem.com
Multiplate®	ASPItest	Dynabyte; www.Multiplate®.net
	ADPtest	Dynabyte; www.Multiplate®.net
	PGE1	Dynabyte; www.Multiplate®.net
	TRAPtest	Dynabyte; www.Multiplate®.net
	RISTOtest	Dynabyte; www.Multiplate®.net
VASP	PLT/VASP/P2Y12	BioCytex; www.biocytex.fr

2. ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
AS	average size
AU	aggregation units
au	area under the curve
AvWS	acquired von Willebrand syndrome
BSS	Bernard-Soulier syndrome
BT	bleeding time
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
COL	collagen
COX-1	cyclooxygenase-1
CPA	cone and plate(let) analyzer
CT	closure time
EPI	epinephrine
ESRF	end stage renal failure
FITC	fluorescein isothiocyanate
GP	glycoprotein
GT	Glanzmann thrombastenia
H	high

HS	high sensitive
IRonI	Impact-R™ wells on Impact™
L	low
LTA	light transmission aggregometry
MACE	major adverse cardiac events
max.AMP	maximal aggregation
MI	myocardial infarction
PFA	platelet function analyzer
PGE_1	prostaglandin E_1
PGH_2	prostaglandin synthetase
PLT	platelet
POC	point-of-care
PPP	platelet poor plasma
PRI	platelet reactivity index
PRP	platelet rich plasma
RISTO	ristocetin
SC	surface coverage
ST	screening test
STEMI	ST-segment-elevation acute myocardial infarction
TI	thrombin inhibitor
TRAP	thrombin receptor activating peptide
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
VASP	vasodilatator-stimulated phosphoprotein
vWD	von Willebrand disease
vWF	von Willebrand factor
vWF:AG	vWF antigen
vWF:RiCoF	vWF ristocetin cofactor
WBA	whole blood impedance aggregometry
WBC	whole blood count

3. Examples of graphs and pictures

Examples of graphs and pictures showing results obtained with LTA (Figure III), Impact-R™ (Figure IV) and Multiplate® (Figure V)

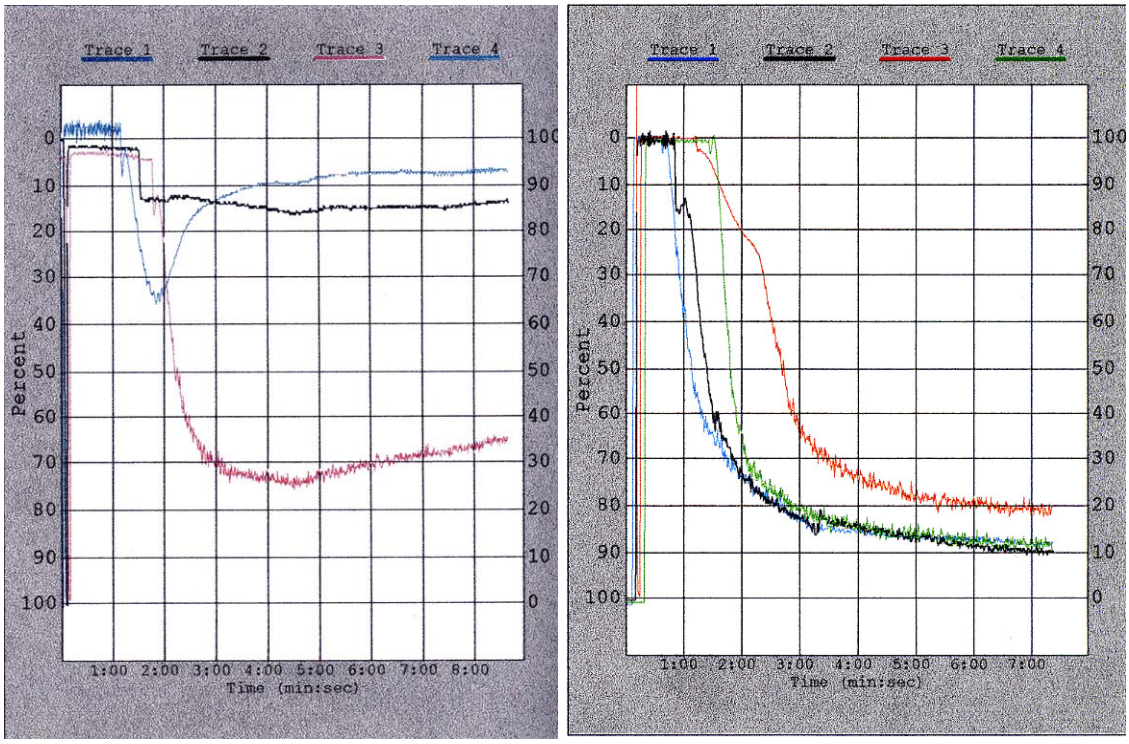


Figure III: LTA graphs of a patient on anti-platelet therapy (left) and of a healthy control (right)
The figure shows graphs obtained with the LTA. Left: indicating good response to Clopidogrel (blue) and Aspirin (black), positive control TRAP (red). Right: Strong aggregation with all agonists: ADP (blue), AA: (black), EPI (red), TRAP (green)

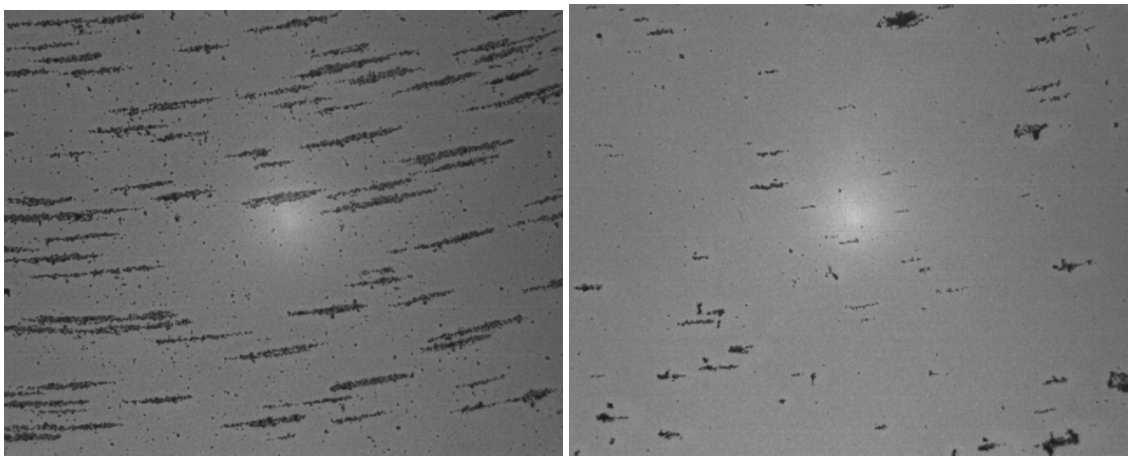


Figure IV: Pictures of the microscopic analysis on Impact-R™ (AA-response tests) of patients on anti-platelet therapy

The figure shows pictures of the microscopic analysis on Impact-R™, indicating good response (SC 9%) (left) and non-response to Aspirin (SC 1,9%) (right)

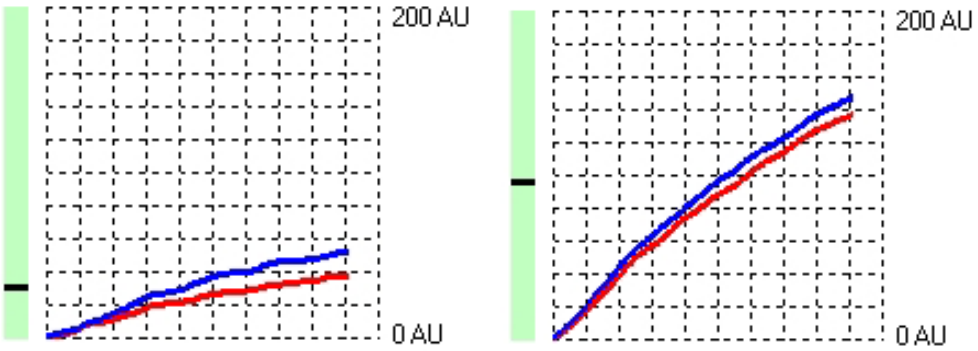


Figure V: Graphs obtained with the Multiplate® ADPtest of patients on anti-platelet therapy

The left picture shows good response to Cloidogrel (23 AU) while the right picture shows results indicating Clopidogrel resistance (73 AU).

VIII. CURRICULUM VITAE

Martina Leitner
Hadikgasse 118/8
1140 Wien
T: 0650/4758591
martina_leitner@gmx.at

Persönliche Daten:

Geburtstag: 23.April 1975
Geburtsort: Wien
Familienstand: ledig

Ausbildung:

1980 – 1993	Volksschule und Mittelschule in Linz und Wels
1994 – 1997	Schule für den medizinisch-technischen Fachdienst, AKH Linz
1998 – 2000	WIKURG für Berufstätige in Graz, Matura
2001 – 2009	Studium der Humanbiologie (Humangenetik), Universität Wien

Diplomarbeit:

2007 – 2009	"Evaluation of Laboratory Methods for Testing Anti-Platelet Drug Resistance" Medizinische Universität Wien, Universitätsklinik für Blutgruppenserologie und Transfusionsmedizin

Berufliche Tätigkeiten:

1997 – 2001	Ordination Dr. Martin Hoff, Graz
2000 – 2001	Sanatorium Dr. Hoff, Graz
2005 – 2006	An der Grub Bio Research GmbH

Wien, im März 2009