

Clinical implications of monitoring the efficacy of  
antiplatelet therapy in patients after percutaneous  
coronary intervention

Ph. D. Thesis

*by*

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# 1. Abbreviations

ACS	Acute Coronary Syndrome
Agg <sub>max</sub>	Maximal Platelet Aggregation
Agg <sub>late</sub>	6-minute Late Platelet Aggregation
ARC	Academic Research Consortium
CES1	Cholin Esterase-1
COX	Cyclo-Oxygenase
CYP450	Cytochrome P450 Enzyme System
DES	Drug-Eluting Stent
disAgg	Platelet Disaggregation
GPI	Glycoprotein IIB/IIIa inhibitor
HPR	High Platelet Reactivity
HPPR	High Post-Treatment Platelet Reactivity
ISR	In-stent Restenosis
LD	Loading Dose
LTA	Light Transmission Aggregometry
MACE	Major Adverse Cardiac Events
MFI	Mean Fluorescence Intensity
MI	Myocardial Infarction
NSTEMI	Non-ST-Elevation Myocardial Infarction
PCI	Percutaneous Coronary Intervention
PDGF	Platelet Derived Growth Factor
PPP	Platelet-Pure Plasma
PRP	Platelet-Rich Plasma
PRI	Platelet Reactivity Index
ST	Stent Thrombosis
sP-selectin	Soluble P-selectin
sVCAM-1	Soluble Vascular Cell Adhesion Molecule-1
TFPCI	Transfemoral Percutaneous Coronary Intervention
TLR	Target Lesion Revascularisation
TRPCI	Transradial Coronary Angioplasty
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
VASP	Vasodilator Stimulated Phosphoprotein

## 2. Introduction

Percutaneous coronary interventions (PCI) have revolutionized the treatment of ischemic heart disease. Following the pioneer interventions of Andreas Grüntzig in 1977, approximately 10.000 patients are treated with this technique every year in Hungary. Exploiting numerous advantages of a minimal invasive intervention, substantial technological and pharmacological advances were made to increase the feasibility and procedural success during the last decade. Consequently, balloon angioplasty, the initial method for dilating coronary stenoses has been replaced with coronary stent implantation due to the lower rates of acute (dissection, abrupt vessel closure) and chronic (restenosis) complications of the latter. (1, 2) Although stent implantation dramatically improved the overall success rate of PCI, limitations are also evident both in short- and long-term. The main shortcomings include stent thrombosis (ST) and in-stent restenosis (ISR) that both result in target-vessel failure after PCI.

### 2.1 Stent thrombosis

Stent thrombosis is an acutely occurring, quick and mostly total occlusion of a previously implanted coronary stent that usually manifests in high-mortality myocardial infarction (MI). (3) In parallel to de novo coronary artery thrombosis, stent thrombus is mainly formed by activated and aggregated platelets that are stabilized by fibrin clots. It is thought to be a multifactorial event, in that both procedural (under-expansion, malposition and malapposition of stents, long stented segment, residual edge-dissection), clinical (low ejection fraction, acute myocardial infarction, impaired renal function, diabetes) and hemorrheologic abnormalities (premature discontinuation of antiplatelet therapy, aspirin/thienopyridine resistance) play substantial role. (4) Notably, the administration of dual antiplatelet therapy has decreased the occurrence of ST compared to aspirin monotherapy or aspirin plus anticoagulation in patients

after PCI. (5-8) Thus, aggressive inhibition of platelet function seems essential to obviate these ischemic events.

Although being a relatively rare event, the prevalence of ST varied largely between clinical trials and registries due to terminological heterogeneity. For obtaining universal definitions and in order to improve comparability and interpretation of results, the Academic Research Consortium (ARC) proposed consensus categories for ST, based on the event probability and level of evidence. Also known as the Glasgow-classification, these criteria define three categories of ST (ARC I, II and III). Alternatively, ST can be classified based on the timing of occurrence. (Table 1) According to the ARC criteria, definite ST occurs in 0.5-1.5% after the first year of PCI, while possible ST has a prevalence of 2 to 4% (9, 10).

TABLE 1. Universal definition of stent thrombosis according to timing and ARC criteria

<b>Academic Research Consortium (ARC) definitions</b>	
ARC-I. Definite:	<ul style="list-style-type: none"> <li>• Angiographic confirmation based on TIMI flow with at least 1 of the following criteria fulfilled within a 48-hour window:               <ul style="list-style-type: none"> <li>–new acute onset of ischemic symptoms at rest</li> <li>–new ischemic electrocardiogram changes that suggest acute ischemia</li> <li>–typical rise and fall in cardiac biomarkers as evidence for an acute MI</li> </ul> </li> <li>• Confirmation of recent ST either at autopsy or via examination of tissue retrieved after thrombectomy</li> </ul>
ARC-II. Probable:	<ul style="list-style-type: none"> <li>• Any unexplained death within the first 30 days</li> <li>• Any MI which is related to acute ischemia in the territory of the implanted stent without angiographic confirmation of stent thrombosis and in the absence of any other obvious cause</li> </ul>
ARC-III. Possible:	<ul style="list-style-type: none"> <li>• Any unexplained death from 30 days after intracoronary stenting until end of trial follow-up</li> </ul>
<b>Timing</b>	
Early:	0 to 30 days after stent implantation
- Acute:	First 24 hours
- Subacute:	From the first to the 30th day
Late:	From the 30th day to 1 year after stent implantation
Very Late:	After 1 year of stent implantation

## 2.2 Instent restenosis

Instent restenosis (ISR) is a chronic process that progressively and irreversibly reduces the previously stented vessel lumen. Similarly to ST, ISR is also a multi-factorial event, in that procedural (under-expansion, malposition, malapposition of stents, long stented segment, smaller stent diameter), clinical (acute myocardial infarction, diabetes, inflammation and hypersensitivity) and hemorrheologic abnormalities (slow flow, not good outflow from stented segment) might play significant part. (11, 12) Compared to normal neointimal formation, ISR is accompanied by overproliferation of the endothelial and smooth muscle cells around the stented segment that leads to a fibrous and thickened neointima. (13, 14) In most of the cases, ISR develops in the first year after coronary intervention and is usually manifested in recurrent stable angina. However, in a minority (2-3%) of the patients, ISR leads to myocardial infarction due to the critical ischemia. (15) Recently, the role of platelet activation and impaired response to antiplatelet therapy were also suggested in the development of ISR as activated platelets might trigger the proliferation of the neointimal tissue by releasing growth factors (PDGF) and recruiting leucocytes. (13)

With the implantation of bare metal or cobalt chromium stents, ISR occurred in 20 to 40% of patients after PCI. In the past years, drug-eluting stents (DES) proved to be extremely successful in preventing instent restenosis. (9, 10) The anti-inflammatory, anti-proliferative and/or cytostatic drugs that are released from stents to the vessel wall were successful to prevent neointimal hyperplasia and decreased the incidence of angiographic instent restenosis below 10%. On the other hand, the eluted antiproliferative drugs prevent healing and complete endothelization of such stents that prolongs the risk of stent thrombosis. Likewise, efficient and long-lasting antiplatelet therapy has a substantial importance in the DES era, as well. Despite being successful in decreasing the rate of target lesion revascularisations (TLR), the first-generation drug- (sirolimus and paclitaxel) eluting stents failed to reduce hard

cardiovascular endpoints, such as cardiac death or MI compared to bare metal analogues after PCI. Moreover, higher prices also limit their availability. As the current penetration of DES does not exceed 20-25%, ISR remains a clinically relevant problem in Hungary.

## 2.3 Antiplatelet agents

Platelets play essential role in the process of atherothrombosis and in progression of arteriosclerosis. (13, 16) As they also contribute to the development of adverse cardiovascular events after PCI, inhibition of platelet activation and aggregation is one of the major pharmacological goals in our therapeutic regimen. Currently, three main groups are available that inhibit platelet activation and/or aggregation.

### 2.3.1 Cox-1 inhibition: Aspirin

Cyclooxygenase (COX) enzyme converts arachidonic acid to a series of prostanoids, most importantly thromboxane A<sub>2</sub> (TXA<sub>2</sub>) that activates platelets on its specific surface receptors. As TXA<sub>2</sub> is a major platelet activator, aspirin, an irreversible COX-1 antagonist is an efficient platelet inhibitor. (Figure 1) Currently, three COX isoenzymes are known (COX-1, COX-2 and COX-3); however platelets only express COX-1 constitutively. As platelets are lack of nucleus thus not capable of active protein synthesis, irreversible inhibition of COX-1 enzyme leads to a loss of COX-1 activity for the whole lifespan of the affected platelet.

Clinical benefits of aspirin therapy have been proven in various subsets of patients with ischemic heart disease. Compared to the lack of antiplatelet therapy, aspirin reduced the outcome of any serious vascular event by about one quarter, non-fatal myocardial infarction by one third, and vascular mortality by one sixth according to a large meta-analysis comprising 135 000 patients. (17) Likewise, it is recommended lifelong after PCI. As daily



doses above 150 mg did not increase the efficacy but the occurrence of gastro-intestinal side-effects and bleeding, low-dose therapy (75-150 mg) it is recommended for chronic administration. (18, 19)

### 2.3.2 ADP-receptor antagonists

Thienopyridines bind to P2Y<sub>12</sub> ADP receptor, one of the key initiators of platelet activation. (20, Figure 2) Ticlopidine was the first-generation oral thienopyridine that was replaced by clopidogrel due to the quicker onset of action and lower rates of hematopoietic side effects. Clopidogrel is a pro-drug that is metabolized in almost 75% to an inactive metabolite by blood esterases (CES1) after gastro-intestinal absorption. (20) Likewise, only one quarter of the prodrug is available for hepatic activation through the cytochrome P450 (CYP) enzyme system. The activation of clopidogrel is a two-step process: first, oxidation to 2-oxo-clopidogrel, then hydrolysis for a thiol derivate that irreversibly binds to P2Y<sub>12</sub> receptor. (20) These processes are mainly catalyzed by the CYP2C19 and to a lesser extent by CYP3A5, CYP3A4, CYP2C9, CYP1A2 and CYP2B6 isoenzymes. (21) The efficacy of the bioactivation of clopidogrel is decreased in many patients as the Caucasian population carries a loss-of-function allele of the CYP2C19 isoenzyme in approximately 30%. (22) Moreover, drug interactions on this enzyme might also impair active metabolite generation. (23)

The onset of action of a 75-mg clopidogrel requires approximately 10 to 12 hours. To achieve a quicker onset of action it is possible to give an oral bolus (recently 600 mg) that achieves a measurable effect at 2 hours and has a peak effect at 4-6 hours.

The value of clopidogrel therapy is well established in a wide spectrum of patients with ischemic heart disease. Clopidogrel has been proven to be superior to aspirin in a high-risk subset of stable angina patients (post-MI, peripheral artery disease) a in the CAPRIE trial. (24)

The CURE study was essential to demonstrate the clinical benefits of supplementing aspirin

monotherapy with clopidogrel in patients with non-ST segment elevation ACS reducing the composite endpoint of cardiovascular death, myocardial infarction or stroke by approximately 20%. (25) Results of the CLARITY and COMMIT trials confirmed that clopidogrel reduced ischemic events in patients with acute myocardial infarction. (26, 27) Notably, these results were later extrapolated to the primary PCI setting; however, no randomized trial ever investigated the value of clopidogrel in patients undergoing PCI for ST-segment elevation MI. However, the need for clopidogrel to reduce ischemic complications in patients undergoing PCI is well represented in the current guidelines that recommend clopidogrel therapy in all stable angina and acute coronary syndrome patients after PCI unless contraindications exist. (18, 19) Notably, a clear increase in bleeding complications was also registered in some trials comparing dual antiplatelet treatment to aspirin monotherapy. (25, 28) These results highlight the fact that tailoring the aggressiveness of antiplatelet therapy is essential to achieve the maximum efficacy with the lowest harm to the patient. This aspect might be discovered in the results of the CHARISMA study, in that dual antiplatelet therapy of aspirin and clopidogrel failed to demonstrate any advantage over aspirin monotherapy in a low-risk, primary prevention group. (28) It is still a matter of debate, how long should clopidogrel be administered after PCI. According to the CREDO and PCI-CURE trials, it is beneficial to prolong clopidogrel therapy beyond 30 days up to 9-12 months after stent implantation. (29, 30) Confirming these findings, premature discontinuation of clopidogrel was associated with high rates of recurrent ischemic events and proved to be a strong and independent predictor of adverse outcome, most importantly stent thrombosis. (31)

There are novel, more potent ADP-receptor antagonists being investigated or under approval, recently. Prasugrel is a third-generation thienopyridine that has demonstrated a significant reduction compared to clopidogrel in the composite outcome of cardiovascular death, MI and stroke among ACS patients in the TIMI38 trial. (32) Ticagrelor is a non-thienopyridine,

reversible, direct-acting P2Y<sub>12</sub> receptor antagonist. It belongs to the cyclo-pentyl-triazolo-pyrimidine (CPTP) class, and was investigated in a large-scale phase III trial (PLATO) among high-risk ACS patients. Compared to clopidogrel, the administration of ticagrelor produced a 16% relative risk reduction in the composite outcome of cardiovascular death, MI or stroke. (33)

### 2.3.3 Glycoprotein IIb/IIIa inhibitors

Contrary to aspirin and thienopyridines, GPIIb/IIIa inhibitors (GPI) are intravenously administered highly potent and promptly-acting antiplatelet agents that block the fibrinogen receptor hence inhibit the final common pathway of platelet aggregation. There are synthetic, small-sized particles (eptifibatide and tirofiban) and monoclonal antibodies (abciximab) that bind to the GPIIb/IIIa receptor. The role of GPIs is well established in ACS, mostly in troponin-positive and diabetic patients. (34-36) However, they also increased the rate of bleeding events. (37) Based on their net clinical benefit, they are recommended in patients with acute myocardial infarction admitted for primary PCI to decrease early thrombotic complications. Notably, they failed to prove clinically relevant benefit in the stable angina population, and only bail-out conditions (slow flow, no-reflow, visible thrombus) indicate their use during elective PCI. (38) Contrary to intravenous molecules, administration of oral GPIs paradoxically increased mortality and ischemic events in preliminary trials. (39)

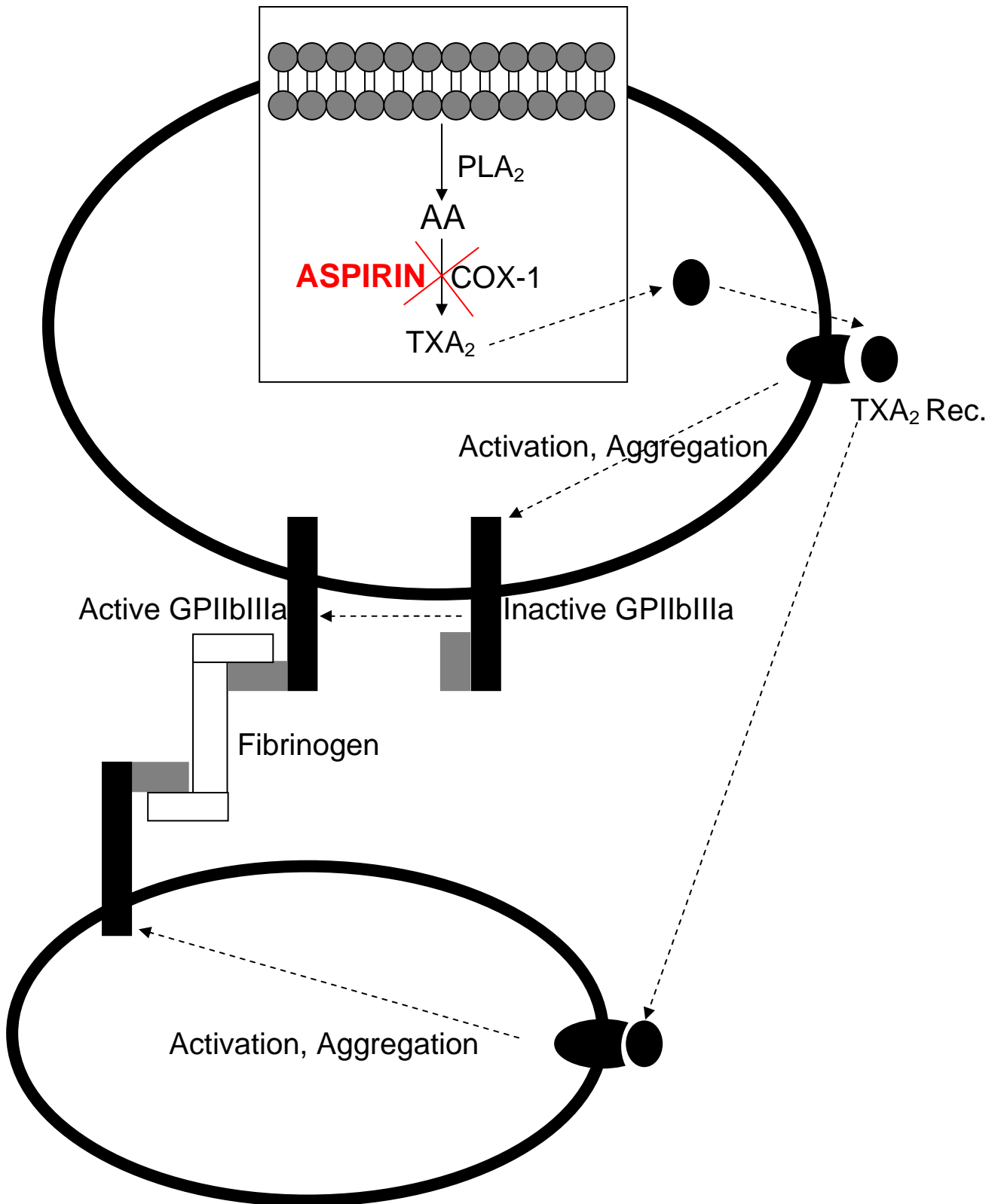


Figure 1. Mechanism of aspirin action in the platelet. PLA<sub>2</sub>: Phospholipase A<sub>2</sub>; AA: Arachidonic Acid, COX-1: Ciclo-oxigenase 1; TXA<sub>2</sub>: Thromboxane A<sub>2</sub>.

Clopidogrel, ticlopidine  
prasugrel, ticagrelor

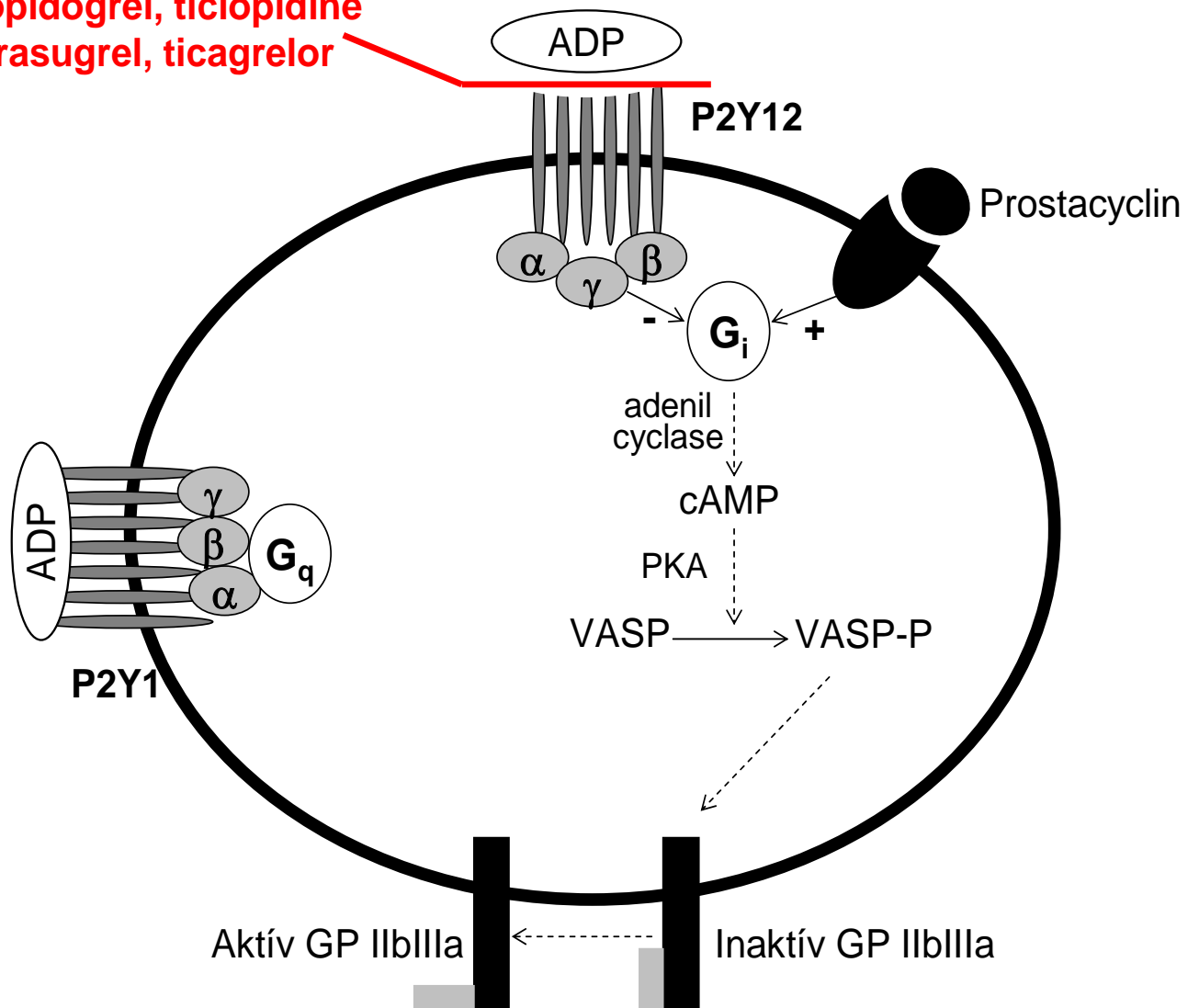


Figure 2. Intracellular signalling of P2Y12 ADP receptor activation in the platelet. ADP has two specific receptors: P2Y12 is coupled to an inhibitory G-protein and P2Y1 is linked to G<sub>q</sub>. PKA: protein kinase A; VASP: vasodilator stimulated phosphoprotein.

## 2.4 Inter-individual differences in response to antiplatelet therapy

According to *in vitro/ex vivo* measurements, administration of aspirin does not inhibit platelet activation and aggregation in a proportion of patients; their platelet reactivity is comparable to aspirin-free subjects. Classified as ‘aspirin resistants’ or ‘non-responders’, these patients can be separated from good responders with laboratory assessments. (40) On the other hand, there are subjects who incur adverse thrombotic events despite being on aspirin therapy that is considered a ‘treatment failure’. Although treatment failure to aspirin and aspirin resistance are different – the first is defined by clinical the latter by laboratory assessment – aspirin resistant patients were shown to be at higher risk for ischemic vascular events. (41) Hence, aspirin resistance might be more than just a laboratory curiosity as high proportion of aspirin resistants ended up with a clinical treatment failure. (42) As a result of methodical and terminological heterogeneity, the prevalence of aspirin resistance varies a lot between authors; ranging from 5% to 65%, with a recent meta-analysis suggesting a mean prevalence of 28%. (43) These results highlight the fact that not all methods are equal in defining antiplatelet response to aspirin. (40, 44)

In patients after PCI, the administration of clopidogrel has dramatically reduced the rate of ischemic events as well as bleeding complications compared to the coumarin and aspirin combination. (5-8) However, thrombotic events still occurred in spite of the dual antiplatelet therapy, thereby extensive research started to explore the individual response to clopidogrel. Early studies demonstrated large inter-individual differences in response to a fix-dose clopidogrel, and similar to aspirin, the term “clopidogrel resistance” was created and widely applied to refer for patients with inappropriate response. (45-47)

Numerous observational studies pointed out the higher risk of patients with inappropriate response to clopidogrel to recurrent thrombotic events. (48-55) However, according to the 2005 ACC/AHA/SCAI guidelines on PCI, antiplatelet testing is only recommended in

patients in whom ST would be of catastrophic consequences (18). This is due to the lack of a uniformly available, standardized, cheap, quick and reliable bedside assay that provides comparable and interpretable results of antiplatelet efficacy. Moreover, results of randomized, sufficiently powered trials are also awaited to clarify the clinically relevant cutoff values. Currently, light transmission aggregometry is considered the historical gold-standard in monitoring the efficacy of antiplatelet therapy. (56)

### 3. Aims

The main aims of our examinations were the following:

- to characterize the individual response and efficacy of both aspirin and thienopyridine therapy in patients admitted for percutaneous coronary intervention and compare the agreement between relevant methods in monitoring antiplatelet efficacy.
- to determine the possible clinical implications of the variability observed in the antiplatelet response among patients after PCI.
- to determine the significance of diabetes mellitus on the prevalence of low response to aspirin and clopidogrel.
- to determine alternative pharmacological approaches in order to overcome inadequate response to clopidogrel and to analyze possible predictors of the response to the alternative approach.
- to determine the prognostic significance of the used access site (femoral vs. radial) on both bleeding and ischemic complications after PCI in patients receiving dual/triple antiplatelet therapy.



## 4. Methods

### 4.1 Light Transmission Aggregometry

Carat TX4 4-channel light transmission aggregometer (LTA, CARAT Diagnostics, Budapest, Hungary) was used for *ex vivo* measurements to monitor the efficacy of antiplatelet therapy. LTA registers optical density of a suspension trans-illuminated with infrared light. The higher the optical density of the suspension, the less light reaches the sensor. LTA dynamically measures and displays infrared light intensity during assessment for 7 minutes. Having introduced in the late '60-ies, LTA became the gold-standard of measuring platelet aggregation in subjects with platelet function disorders and in patients receiving antiplatelet therapy. The method is relatively cheap, broadly available and well accepted; enables monitoring antiplatelet efficacy selectively with specific agonists. However, measurements are poorly standardized, time-consuming, non-automated, require trained personnel for sample preparation and measurement. As a conclusion, it is difficult to compare results and generalize consequences obtained with this method.

For assessments, 10 ml blood needs to be drawn into vacuum-tubes anticoagulated with 3.8% sodium-citrate from every patient. For getting platelet-rich plasma (PRP), blood is centrifuged at 2000 rpm for 4 minutes. Further centrifugation for 10 minutes at 4000 rpm results in platelet-poor plasma (PPP). At baseline, PPP is used to set 100%, while PRP 0% light transmission on the aggregometer. Then, platelet-specific agonists are added into PRP to stimulate platelet aggregation with a continuous magnetic stirring at 37 degree Celsius. In antiplatelet-free subjects, activation of resting platelets results in formation of platelet aggregates that decrease optical density of the plasma. Thus, light transmission increases steeply after the injection of the agonist forming a plateau thereafter. Platelet reactivity is usually expressed with the maximal platelet aggregation value ( $Agg_{max}$ ) of the registered

optical curve, while other parameters (late aggregation [Agg<sub>late</sub>], steepness of slope, area under curve [AUC]) and disaggregation [disAgg] may also be determined. (Figure 3, 4)

Efficient antiplatelet therapy prohibits platelet activation, limiting the formation of platelet aggregates and decreasing the peak value of the aggregation curve. Likewise, high Agg<sub>max</sub> values are typical for untreated subjects and low responder patients, while low Agg<sub>max</sub> reflects effective platelet inhibition. (Figure 3, 4)

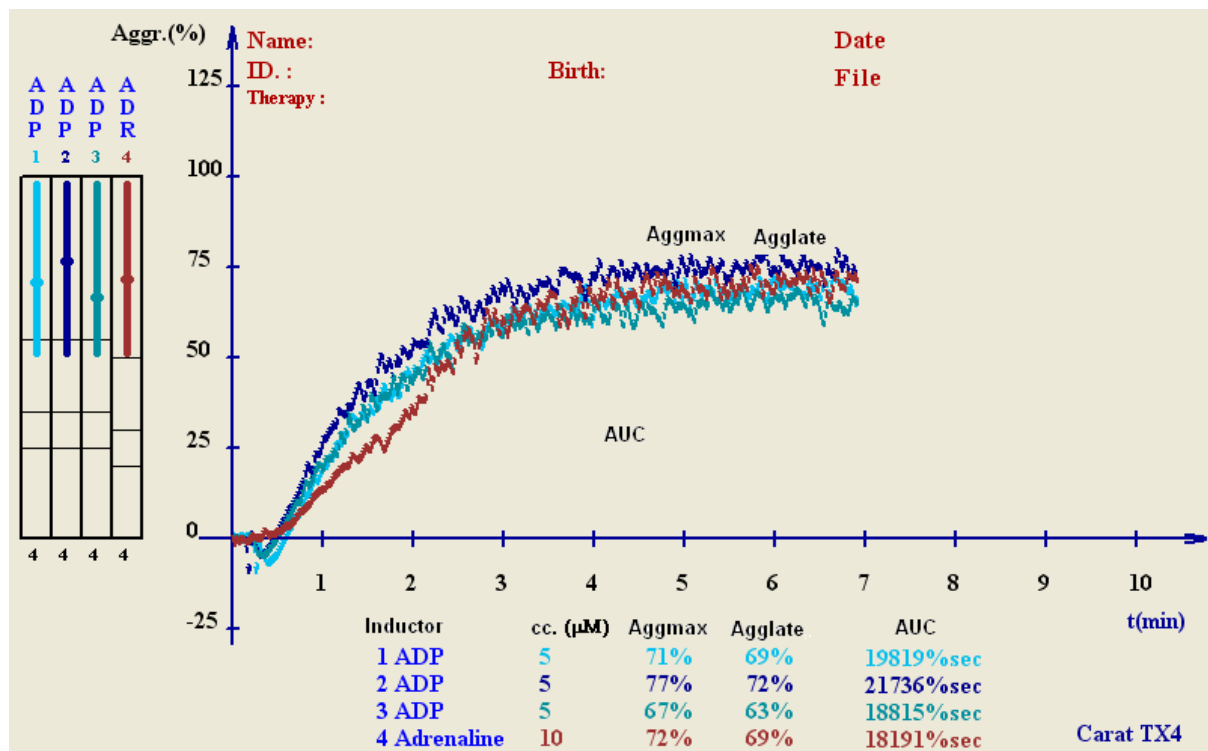


Figure 3. Light transmission assessment with ADP 5 µM and adrenaline 10 µM in a patient not exposed to antiplatelet therapy. Agg<sub>max</sub>: maximal aggregation, Agg<sub>late</sub>: 6-minute late aggregation, AUC: area under the curve. AUC is calculated as the sum of the actual aggregation values in every second from agonist addition until 6 minutes; divided by 100. Disaggregation was defined according to  $(Agg_{max} - Agg_{late}) / Agg_{max} \times 100$ . Disaggregation might be calculated as  $(Agg_{max} - Agg_{late}) / Agg_{max} * 100$ .

As antiplatelet agents block a specific pathway of platelet activation, their efficacy can be measured with a specific agonist: ADP is used to test the efficacy of thienopyridine therapy, while adrenaline, collagen or arachidonic acid is suitable to measure efficacy of aspirin treatment. This means one of the most important advantages of the assay, i.e. to measure the efficacy of antiplatelet agents using specific agonist with high selectivity.

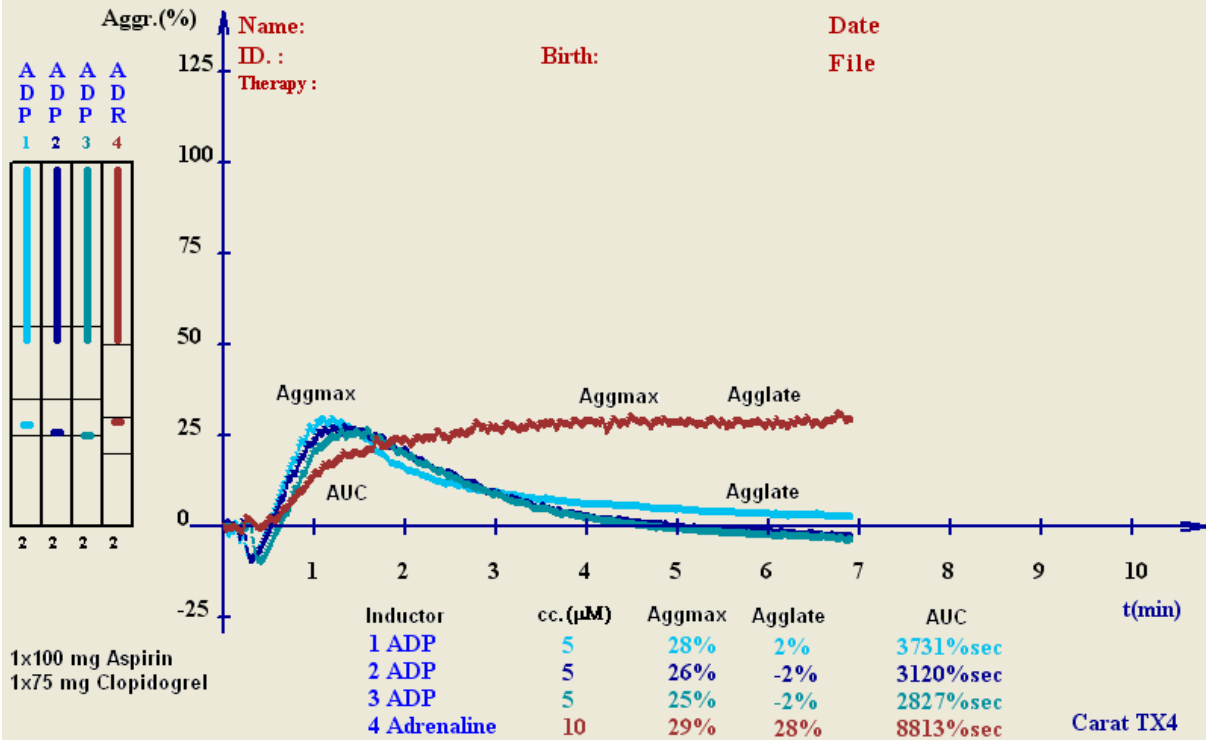


Figure 4. Light transmission assessment with ADP 5 μM and adrenaline 10 μM in a patient with good response to aspirin and clopidogrel therapy. Aggmax: maximal aggregation, Agglate: 6-minute late aggregation, AUC: area under the curve. AUC is calculated as the sum of the actual aggregation values in every second from agonist addition until 6 minutes; divided by 100. Disaggregation was defined according to  $(Aggmax - Agglate) / Aggmax \times 100$ . Disaggregation might be calculated as  $(Aggmax - Agglate) / Aggmax \times 100$ .

## 4.2 Vasodilator Stimulated Phosphoprotein (VASP)

Platelets express two subtypes of ADP surface receptors: while P2Y1 is essential for initiation of platelet activation, stimulation of P2Y12 receptor causes a decrease in adenylyl-cyclase resulting in lower levels of intracellular cyclic adenosine monophosphate (cAMP). Cyclic AMP is necessary for phosphorylation of a second messenger known as vasodilator-stimulated phosphoprotein (VASP). VASP is important for regulation of the cytoskeleton and for conversion of glycoprotein IIb/IIIa to its active conformation, thus permitting platelets to aggregate. VASP exists in both phosphorylated and dephosphorylated states. The phosphorylated form is characteristic for a resting platelet. Inhibition of cAMP-activity after activation of the P2Y12 receptor by ADP leads to an increase in VASP dephosphorylation, whereas blockade of P2Y12 receptor by the active metabolite of a thienopyridines inhibits dephosphorylation. Thus, the ratio of phosphorylated to dephosphorylated VASP reports the degree of the P2Y12 receptor blockade. (Figure 5)

Currently, flow cytometric measurement of VASP phosphorylation represents the most specific method for monitoring the efficacy of thienopyridine therapy. However, the kit is expensive, the process is time-consuming, requires special instrumental and personal training giving very limited availability to the assay.

In our measurements, the phosphorylation status of VASP was analyzed with fluorescent antibody against VASP-P on a Beckmann Coulter flow cytometer using PLT VASP/P2Y12 kit (Biocytex, Marseille, France). For this assay, citrated whole blood was incubated with PGE1 with or without ADP. After fixation with paraformaldehyde, the cells were permeabilized and incubated with a primary mouse monoclonal antibody specific for phosphorylated VASP, followed by a secondary fluorescein isothiocyanate (FITC)-conjugated polyclonal goat-antimouse antibody. Samples were then analyzed by flow cytometry to measure the level of phosphorylated VASP. The efficacy of ADP-receptor inhibition is

expressed with the platelet reactivity index (PRI), which is calculated from the corrected mean fluorescence intensity (MFI) of the PGE1 and the PGE1+ADP-incubated samples as follows:  $PRI = (MFI_{PGE1} - MFI_{PGE1+ADP}) / MFI_{PGE1} \times 100$ . (Figure 5-7)

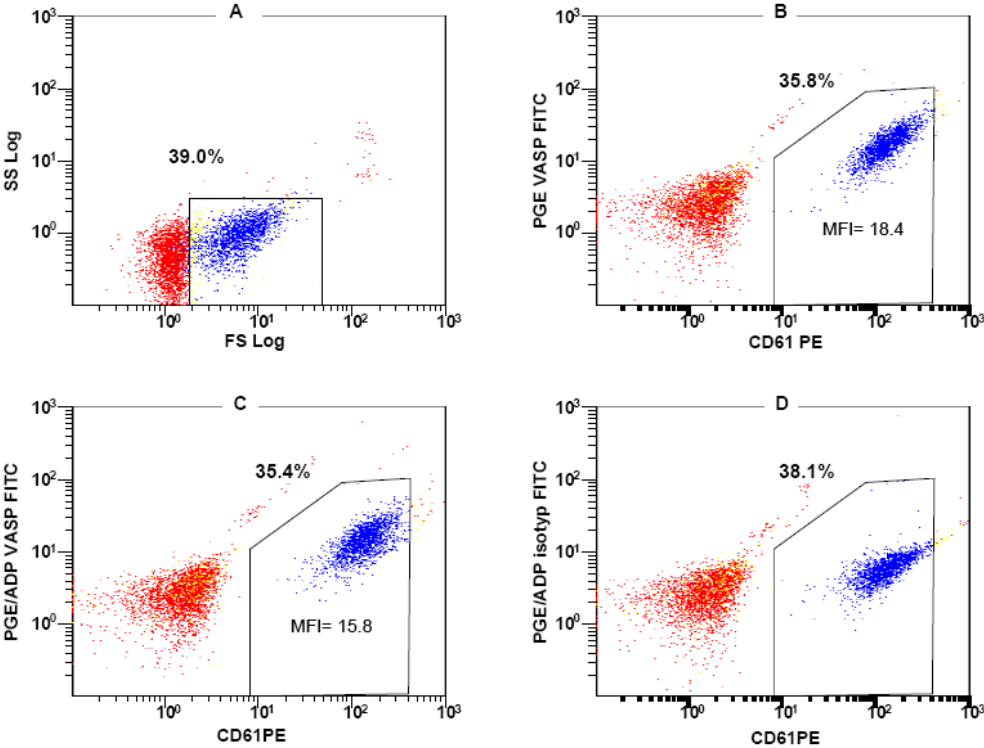


Figure 5. Flow cytometric measurement of VASP platelet reactivity index (PRI) in a patient with good ADP P2Y12 receptor inhibition.  $PRI = (MFI_{PGE1} - MFI_{PGE1+ADP}) / MFI_{PGE1} \times 100$ . PRI is 14.13% in this case, suggesting almost full receptor saturation.

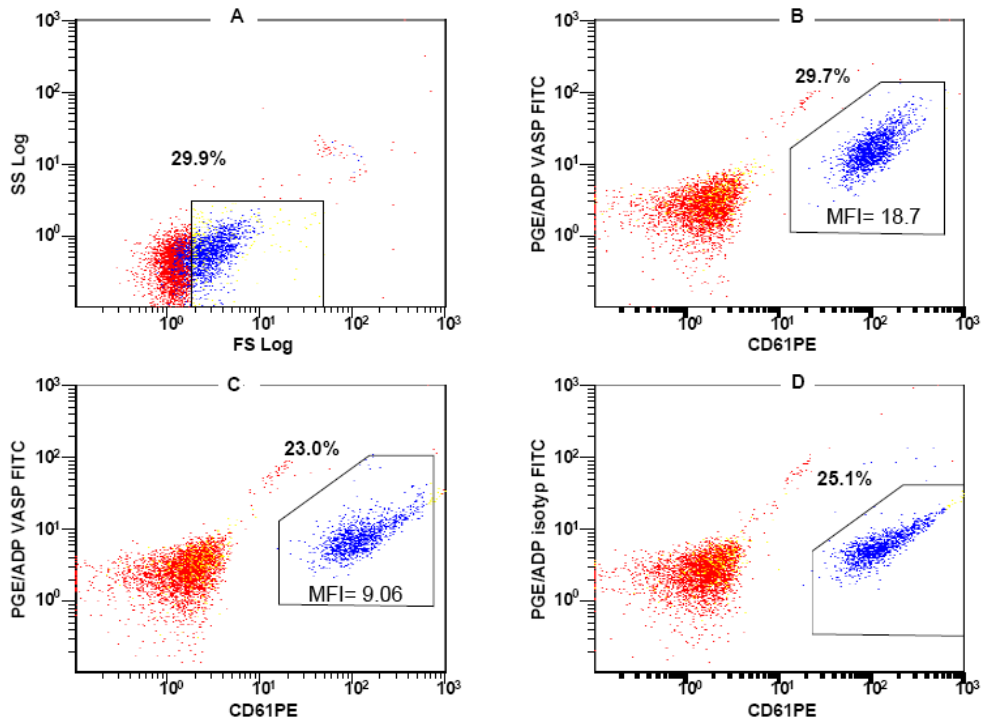


Figure 6. Flow cytometric measurement of VASP platelet reactivity index (PRI) in a patient with poor ADP P2Y12 receptor inhibition.  $PRI = (MFI_{PGE_1} - MFI_{PGE_1+ADP}) / MFI_{PGE_1} \times 100$ . PRI is 51.55%, showing high platelet activation through the ADP-receptor.

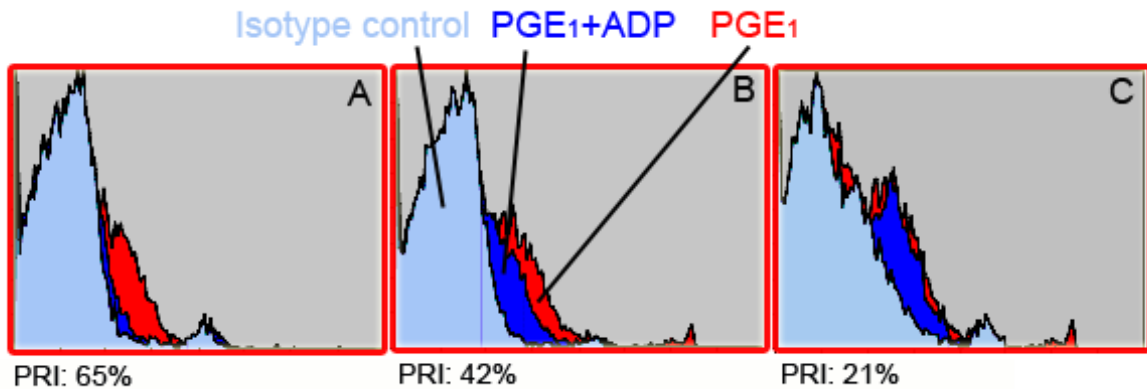


Figure 7. Histograms of mean fluorescent intensities (MFI) after incubation with PGE<sub>1</sub> alone and PGE<sub>1</sub>+ADP in VASP assay. Panel A depicts result in a patient with high VASP dephosphorylation after ADP incubation, indicating low P2Y12 ADP receptor inhibition and consequently high platelet reactivity (PRI: 65%). On the other hand, patient C shows the same MFI value after co-incubation with ADP, reflecting complete ADP receptor inhibition and low platelet reactivity (PRI: 14%). Panel B shows an intermediate case with borderline efficacy of ADP receptor inhibition.  $PRI = (MFI_{PGE_1} - MFI_{PGE_1+ADP}) / MFI_{PGE_1} \times 100$ .

### 4.3 Soluble markers of platelet activation

We collected blood samples for examination of soluble markers of platelet activation using Multiplex Fluorescent Bead Immunoassay (Bender MedSystems GmbH, Vienna, Austria). The assay enabled the detection of soluble VCAM-1, soluble CD40L and soluble P-selectin in patients after coronary stent implantation. 5 ml blood was drawn with direct venipuncture, and centrifuged for separating plasma. Samples were immediately frozen to -20°C to avoid loss of bioactive markers. Prior to assay, the frozen plasma was brought to room temperature slowly, and added to the mixture of seven microbead clusters coated with different monoclonal antibody against the intent-to-measure activation markers (sVCAM-1, sP-selectin and sCD40L). The activation markers present in the sample bind to the antibodies adsorbed to the fluorescent beads. Phycoerythrin (PE) conjugated second antibody mixture is added and the specific antibodies bind to the activation markers captured by the first antibodies. Microbead clusters were identified according to their intrinsic far red fluorescent activities (690nm). Rows of standard dilution of the intent-to-measure markers were created in parallel to sample analysis in order to obtain standard curves of PE fluorescent intensity (575nm) of the adequate bead cluster. Far red fluorescent intensity (indicate the bead cluster, the activation marker) and PE fluorescent intensity (indicate the concentration of the activation marker in the sample) of the microbeads was read on FACSCalibur flow cytometer. Plasma levels of activation markers were calculated using standard dilution curves of mean fluorescent intensity (MFI).

## 5. Experimental results

### 5.1 Monitoring P2Y<sub>12</sub> receptor inhibition with light transmission aggregometry: a comparison with vasodilator stimulated phosphoprotein phosphorylation assay

#### Background:

Clopidogrel acts by blocking the purinergic P2Y<sub>12</sub> ADP-receptor, one of the most important pathways in platelet activation. (13, 20) Unfortunately, the relatively slow onset of action and the high interindividual variability in efficacy are important shortcomings of clopidogrel that are largely driven by the ineffective and highly unpredictable generation of its active, thiol metabolite. (20) The clinical relevance of interindividual differences in response to a fixed-dose therapy has been demonstrated by several observational studies highlighting that the selection of patients with inappropriate P2Y<sub>12</sub> receptor inhibition would be of great importance. (48-55) Due to the lack of a generally accepted, automatized, cheap and standardized bedside test to select such patients, routine screening is not yet recommended. (18, 19) Measuring ADP-stimulated platelet aggregation with light transmission aggregometry is the historical gold-standard to monitor the efficacy of thienopyridine therapy. (56) However, the optimal platelet aggregation parameter ( $Agg_{max}$ ,  $Agg_{late}$ , AUC or disaggregation, Figure 3, 4) to use is not well elucidated. Most of the laboratories are using the maximum value ( $Agg_{max}$ ) of the ADP-stimulated aggregation curve that is achieved early after agonist addition. However, LTA is not P2Y<sub>12</sub>-specific, as ADP also binds to P2Y<sub>1</sub> receptor. (20) The latter initiates shape change with degranulation leading to an unstable, early-stage platelet aggregation that might result in disaggregation during assessment if the P2Y<sub>12</sub> receptor is blocked. Disaggregation during ex vivo testing is typical when low ADP ( $\leq 5 \mu M$ ) concentrations are used. Based on these, some laboratories prefer to use late



aggregation value believing that aggregation measured at 5-6 minutes after ADP stimulation might better represent P2Y12 receptor inhibition without the influence of P2Y1-activity. (50) However, the superiority of  $Agg_{late}$  over other aggregometry parameters has not yet been proved by an independent, P2Y12-specific assay. Flow cytometric assessment of vasodilator stimulated phosphoprotein (VASP) phosphorylation is a recently developed, completely P2Y12-specific method that is considered the gold standard for measuring P2Y12-receptor inhibition.

### Objective:

We aimed to compare ADP-stimulated light transmission aggregometry to the P2Y12-specific VASP phosphorylation assay in order to analyse the agreement between them. Moreover, we sought to test the hypothesis that  $Agg_{late}$  is superior to other estimates of the LTA measurement in monitoring P2Y12 receptor inhibition.

### Methods:

We prospectively recruited clopidogrel-naïve stable angina patients in whom elective percutaneous coronary interventions were performed. Exclusion criteria were acute coronary syndrome, prior thienopyridine or oral anticoagulant therapy, known contraindication to aspirin or clopidogrel, stroke in the past 6 months, known bleeding disorders or low platelet count ( $<100 \times 10^9/L$ ). All patients received a single loading dose of 600 mg clopidogrel and 300 mg enteric-coated aspirin after coronary angiography, just immediately before PCI, after giving written consent for participation in the study. Radial approach was the preferred access site for PCI, with the administration of 5000 IU of unfractionated heparin (UFH) in the arterial sheath after successful puncture. An additional UFH bolus for PCI to achieve a 70 IU/kg heparin dose was left to the discretion of the operator. Twelve to 18 hours after the 600-

mg loading dose of clopidogrel, 20 ml blood was drawn from each patient from a peripheral vein and put into four 4.5-ml BD Vacuutainer tubes, with 3.8% sodium-citrate for anticoagulation for LTA and VASP assessment. All patients gave written consent for participation, and the study was approved by the local ethics committee.

#### Light transmission aggregometry

LTA measurements were performed as described previously. (Section 4.1) The efficacy of clopidogrel therapy was expressed using ADP 5  $\mu$ M, while epinephrine 10  $\mu$ M was used to refer for the efficacy of aspirin therapy. In a subgroup of patients, PRP was preincubated with MRS2179, a specific inhibitor of the P2Y1 platelet receptor in a final concentration of 100  $\mu$ M to test the influence of P2Y1 receptor inhibition on the recorded platelet aggregation values. Former reports have shown that this concentration produce almost complete inhibition of the P2Y1 receptor. Among these patients, aggregation was recorded with and without MRS2179 addition using ADP 5 and 20  $\mu$ M. Maximal aggregation ( $Agg_{max}$ ), 6-minute late aggregation ( $Agg_{late}$ ), disaggregation (disAGG) and the area under the aggregation curve (AUC) were calculated in every measurement.

#### Vasodilator stimulated phosphoprotein (VASP) phosphorylation

Platelet VASP phosphorylation (VASP-PRI) was determined as described in section 4.2.

#### Statistical analysis

Continuous variables are presented as means  $\pm$ SD. Categorical variables are expressed as frequencies and percentages. Spearman's correlation method was used to correlate platelet function values to VASP-PRI and  $r^2$  values were calculated in the linear regression analysis. Due to serious collinearity of the LTA parameters, the multivariable linear regression model

was used with stepwise method to determine the independent linear predictor of VASP-PRI. The results of the linear regression analysis give the standardized coefficient and the unstandardized coefficient with its 95% confidence interval (95%CI). Bland-Altman agreement plots were generated to show the difference between the two measurements as a function of the average of the two measurements of each sample. This analysis specifically measures bias, which can be defined as a systematic error responsible for either under- or overestimation of a value, and sets limits of agreement which indicate the range of under- or overestimation of one reading in comparison with the other. Bland-Altman comparison requires that both methods measure the same feature on a comparable scale. Both LTA assessments and VASP measures post-treatment platelet reactivity, however, LTA parameters are scaled in different ranges. For the comparison with VASP-PRI in Bland-Altman plots, LTA measurements were normalized to a 0 to 100% scale. Receiver-operator characteristic curve (ROC) analysis was used with VASP-defined high platelet reactivity (HPR) as the dependent variable to estimate the predictive value of the LTA parameters. As disaggregation is in inverse correlation with platelet reactivity, a reciprocal transformation was used before the ROC analysis was performed. The optimal cutoff points were determined according to the threshold with the highest sensitivity and specificity.

Agreement among assays to identify patients with normal or high platelet reactivity was assessed through the  $\kappa$  statistic. A p value  $<0.05$  was considered statistically significant in all analysis. Statistical analyses were performed with SPSSv11.0 (SPSS Inc. Chicago, Illinois) and Graphpad Prism 5.0 trial (Graphpad Software Inc).

## Results

### Impact of P2Y1-receptor inhibition on platelet aggregation values

The effect of specific P2Y1 ADP receptor inhibition with 100  $\mu$ M of MRS2179 was tested on ADP-induced  $Agg_{max}$ ,  $Agg_{late}$ , AUC and disAGG values in 36 patients. Preincubation of PRP with MRS2179 significantly decreased LTA estimates compared to ADP-stimulation alone in case of both ADP 5  $\mu$ M ( $Agg_{max}$ : 25.53 $\pm$ 14.20 vs 8.56 $\pm$ 10.80;  $Agg_{late}$ : 3.28 $\pm$ 16.72 vs. -0.47 $\pm$ 11.10; AUC: 51.96 $\pm$ 48.70 vs 19.45 $\pm$ 28.33; disAGG: 53.67 $\pm$ 21.85 vs 33.40 $\pm$ 18.24;  $p < 0.05$  in all paired comparisons) and 20  $\mu$ M ( $Agg_{max}$ : 33.67 $\pm$ 16.24 vs 20.11 $\pm$ 15.09;  $Agg_{late}$ : 16.61 $\pm$ 21.77 vs 7.75 $\pm$ 16.02; AUC: 87.75 $\pm$ 62.01 vs 49.44 $\pm$ 47.95; disAGG: 41.25 $\pm$ 27.46 vs 38.40 $\pm$ 21.87;  $p < 0.05$  in all paired comparisons). The absolute decrease in  $Agg_{max}$  values was substantially greater in response to MRS2179, while  $Agg_{late}$  showed only a modest reduction indicating that P2Y1 receptor inhibition preliminary influences maximal aggregation values. (Figure 8)

#### Correlation among LTA and VASP measurements

Baseline clinical characteristics of the recruited patients are depicted in Table 2. Eighty-nine VASP and LTA measurements were performed 19 $\pm$ 2 hours after receiving a 600-mg loading dose of clopidogrel and 80 patients were sampled on maintenance-phase, at 25 $\pm$ 2 days after PCI.

After the administration of a 600-mg loading dose of clopidogrel, all platelet function measures demonstrated high interindividual variability in efficacy ( $Agg_{max}$ : 28.8 $\pm$ 14.0;  $Agg_{late}$ : 9.4 $\pm$ 17.5; disAGG: 72.0 $\pm$ 33.3; AUC: 66.8 $\pm$ 52.2; VASP-PRI: 49.9 $\pm$ 22.1) that also persisted in the maintenance period ( $Agg_{max}$ : 30.0 $\pm$ 12.6;  $Agg_{late}$ : 9.2 $\pm$ 16.2; disAGG: 71.4 $\pm$ 30.3; AUC: 68.6 $\pm$ 49.5; VASP-PRI: 48.1 $\pm$ 20.6). Based on the 169 LTA measurements, high correlation was found between the maximal and late aggregation values ( $p < 0.001$ ; Spearman's  $\rho$ : 0.91). When LTA values were compared to VASP-PRI, significant, moderate-strength correlations were registered with AUC showing the highest correlation coefficient to

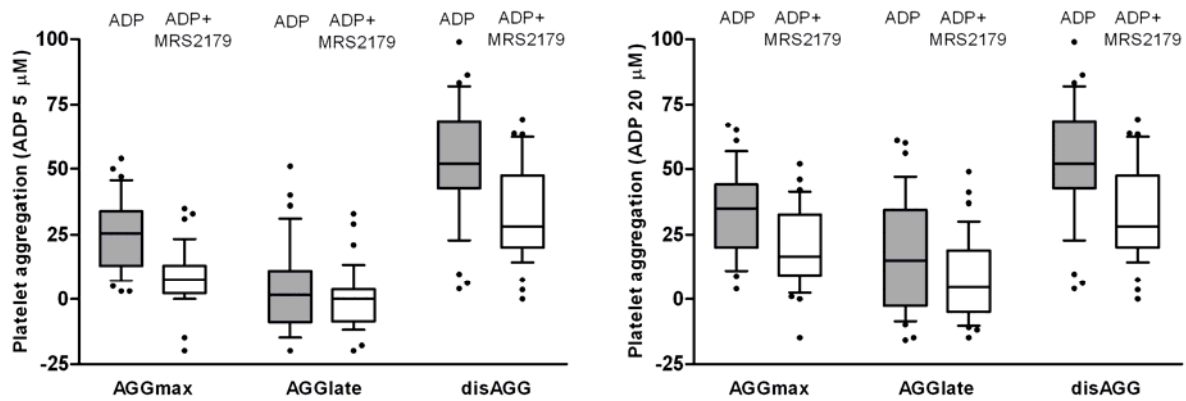


Figure 8. Box and whiskers plots showing the effects of MRS2179, a specific inhibitor of the P2Y1 receptor on ADP-induced platelet aggregation values in 36 clopidogrel-treated patients. The inner lines in boxes indicate mean values with outliers showing 10<sup>th</sup> and 90<sup>th</sup> percentile, respectively. Dots represent individual data out of the 10<sup>th</sup>-90<sup>th</sup> percentile range. Agg<sub>max</sub>: maximal platelet aggregation, Agg<sub>late</sub>: 6-minute late aggregation, disAGG: 6-minute disaggregation. All the paired comparisons have shown significant differences ( $p < 0.05$ ).

VASP-PRI (Agg<sub>max</sub>:  $\rho = 0.48$ ; Agg<sub>late</sub>:  $\rho = 0.48$ ; disAGG:  $\rho = -0.49$ ; AUC:  $\rho = 0.51$ , Figure 9). Notably, the efficacy of aspirin therapy, measured by epinephrine 10  $\mu\text{M}$ , did not correlate to VASP-PRI. ( $p = 0.75$ ,  $\rho = -0.24$ ). Univariate linear regression analysis showed similar relationship between LTA and VASP assessments [Agg<sub>max</sub>: 0.47, 0.75(0.54-0.97); Agg<sub>late</sub>: 0.49, 0.62(0.45-0.78); disAGG: -0.49, 0.34(-0.43- -0.24); AUC: 0.50, 0.21(0.15-0.27)]. To find the independent linear predictor of VASP-PRI, a multivariable linear regression model was used that selected AUC as the independent predictor of VASP-PRI [0.50, 0.21(0.15-0.27)].

Age (years, $\pm$ SD)	61.98 $\pm$ 8.91
Male gender (n, %)	53 (59.6%)
Stable angina (n, %)	89 (100%)
Diabetes (n, %)	28 (31.5%)
INS/OAD/DIET (n, %)	10(11.2%)/ 12(13.5%)/ 6(6.7%)
Hypertension (n, %)	78 (87.6%)
Smoking (n, %)	32 (36.0%)
Current smoker (n, %)	12 (13.5%)
Prior MI (n, %)	14 (15.7%)
Prior CABG (n, %)	6 (6.7%)
PAD (n, %)	4 (5%)
Baseline total cholesterol (mmol/L, $\pm$ SD)	4.00 $\pm$ 0.95
Baseline serum creatinin (mmol/L, $\pm$ SD)	72.56 $\pm$ 16.22
Baseline high sensitivity CRP (ng/ml, $\pm$ SD)	2.28 $\pm$ 1.72
Baseline fibrinogen	3.02 $\pm$ 0.82
Baseline leukocyte count (G/L, $\pm$ SD)	6.87 $\pm$ 1.80
Baseline haemoglobin (g/dl, $\pm$ SD)	129.79 $\pm$ 12.43
Baseline platelet count (G/L, $\pm$ SD)	232.60 $\pm$ 65.67

INS: insulin-treated diabetes mellitus; OAD: oral antidiabetic-treated diabetes; MI: myocardial infarction; CABG: coronary artery bypass grafting; PAD: periferial artery disease.

To adjust for possible interaction of the concomitant aspirin therapy, epinephrine-induced  $Agg_{max}$  values were also added to the model. The adjusted model showed a better fit to the population (R square: 0.34 versus 0.25), and selected AUC as an independent predictor with a higher standardized coefficient [0.66, 0.28(0.22-0.34)]. When the loading- and the maintenance phase outputs of VASP and LTA assessments were analysed separately, similar results were obtained without any difference between the two time points (data not shown).

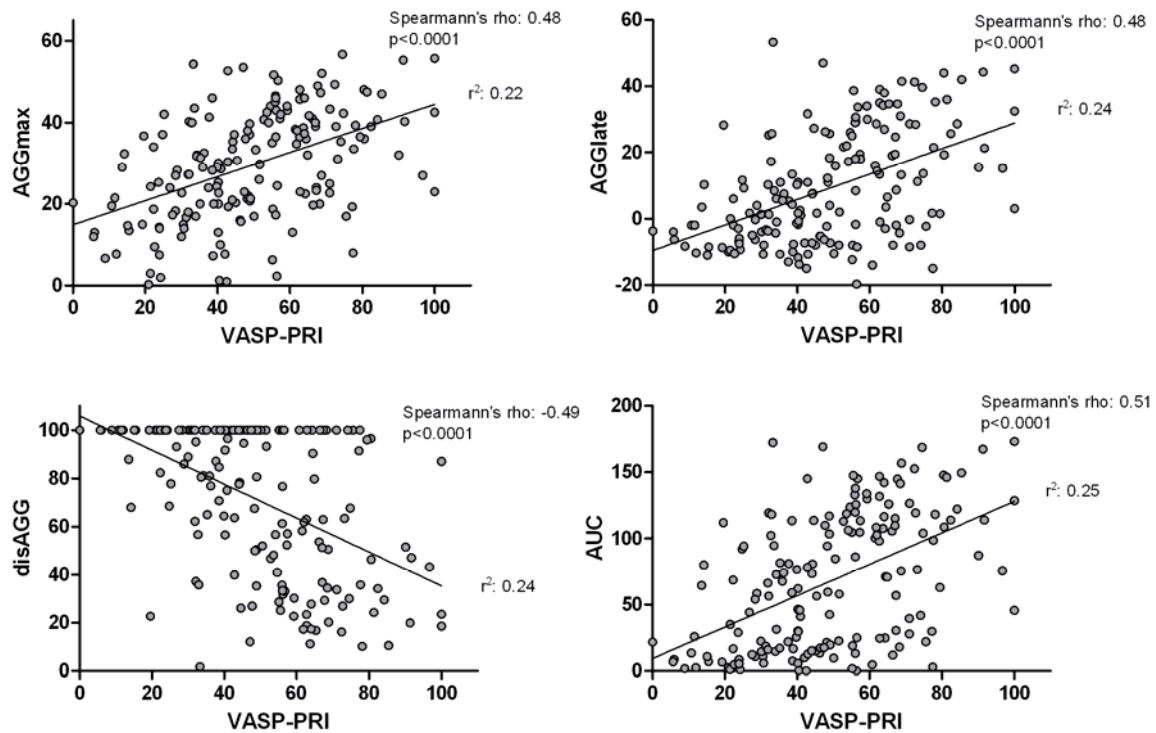


Figure 9. Scatterplots comparing vasodilator stimulated phosphoprotein phosphorylation index (VASP-PRI) and different measures of the light transmission curve. AGGmax: maximal aggregation; Agg<sub>late</sub>: 6-minute late aggregation; disAGG: disaggregation; AUC: area under the light transmission curve.

Bland-Altman plots were used to demonstrate agreement among assays in measuring post-clopidogrel platelet reactivity. (Figure 10) In case of maximal aggregation, the plot showed that platelet reactivity is estimated quite similarly by both methods with significant disagreement in certain individuals (bias: 2.8; limits of agreement: -42.6 - 48.2, range: 90.8). When Agg<sub>late</sub> was plotted against VASP-PRI, the analysis showed that it underestimates platelet reactivity with similar intra-individual differences as Agg<sub>max</sub> (bias: -9.4; limits of agreement: -53.6 - 34.9; range: 88.5). Disaggregation and AUC also underestimated VASP-defined platelet reactivity, with wider disagreement range of the former (bias: -19.8; limits of

agreement: -75.0 - 35.5; range: 110.5 for disAGG and bias: -10.0; limits of agreement: -61.5 - 41.4, range: 102.9 for AUC). The plots also demonstrate that the underestimation of platelet reactivity by  $\text{Agg}_{\text{late}}$ , disAGG and AUC is driven by differences in the low platelet reactivity range (markedly below 50%) where these measures of LTA give significantly lower values of platelet reactivity than VASP-PRI.

#### Agreement between assays in determining normal and high platelet reactivity

The predictive value of LTA variables in determining high platelet reactivity (HPR), defined as a VASP-PRI value greater than 50% was evaluated with receiver-operator characteristic (ROC) curve analysis. (Figure 11) The analysis produced similar predictive values for the examined parameters ( $p < 0.001$  in all cases; area under ROC curve with 95%CI, respectively:  $\text{Agg}_{\text{max}}$ : 0.76, 0.69-0.83;  $\text{Agg}_{\text{late}}$ : 0.75, 0.67-0.83;  $1/\text{disAGG}$ : 0.75, 0.67-0.82; AUC: 0.77, 0.69-0.84). The optimal cutoff values for the LTA estimates were determined with by selecting the threshold with the highest sensitivity and specificity. Based on these cutoff values, the agreement between VASP and LTA estimates in defining normal and high platelet reactivity was analyzed with the  $\kappa$ -statistic. Overall, the agreement was moderate, with  $\text{Agg}_{\text{late}}$  showing the highest  $\kappa$ -value ( $p < 0.001$  in all cases;  $\text{Agg}_{\text{max}}$ :  $\kappa$ :0.45;  $\text{Agg}_{\text{late}}$ :  $\kappa$ :0.53; disAGG:  $\kappa$ :0.51, AUC:  $\kappa$ :0.48). Interestingly,  $\text{Agg}_{\text{max}}$  greater than 32.9% had the highest sensitivity, yet the lowest specificity to identify a patient with VASP-PRI > 50%.



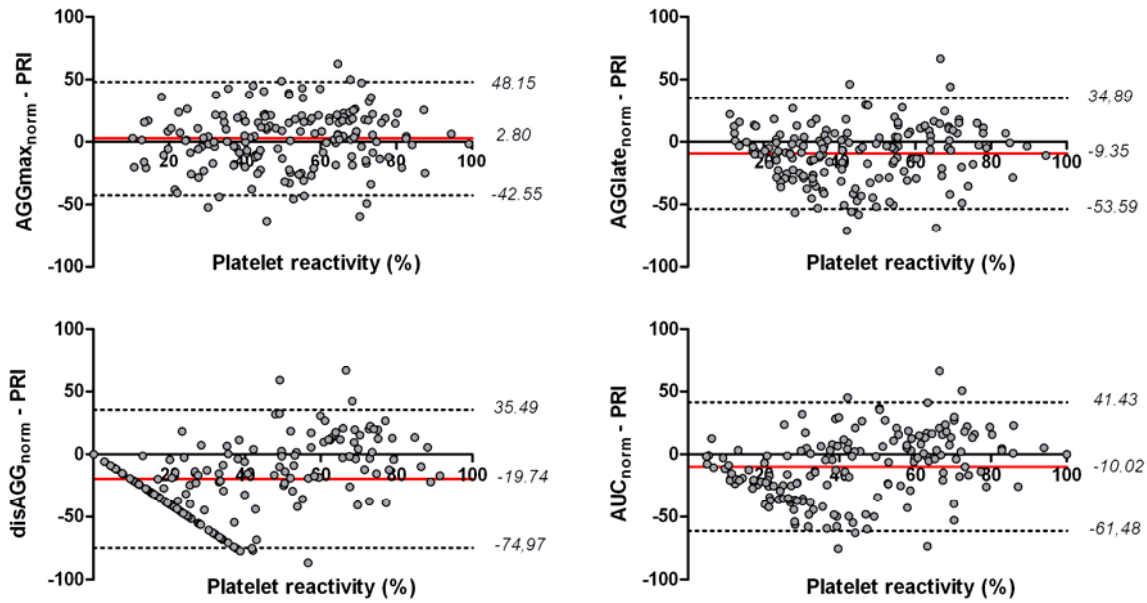


Figure 10. Bland–Altman plots demonstrate intra-individual agreement between different methods measuring the same parameter (platelet reactivity) with limits (dotted lines) depicting the range of variability between the two measures. Bias (red line) is a measure of a systematic error leading to over- or underestimation of a known value (VASP-PRI) by alternative parameters of the light transmission assessment (AGG<sub>max, norm</sub>: normalized maximal aggregation, AGG<sub>late, norm</sub>: normalized 6-minute late aggregation; disAGG<sub>norm</sub>: normalized disaggregation, AUC<sub>norm</sub>: normalized area under the light transmission curve). As the principle of the Bland-Altman analysis is that both measurements evaluate a parameter on a same scale (platelet reactivity %), all the light transmission parameters were normalized to the scale of VASP-PRI (from 0% to 100%).

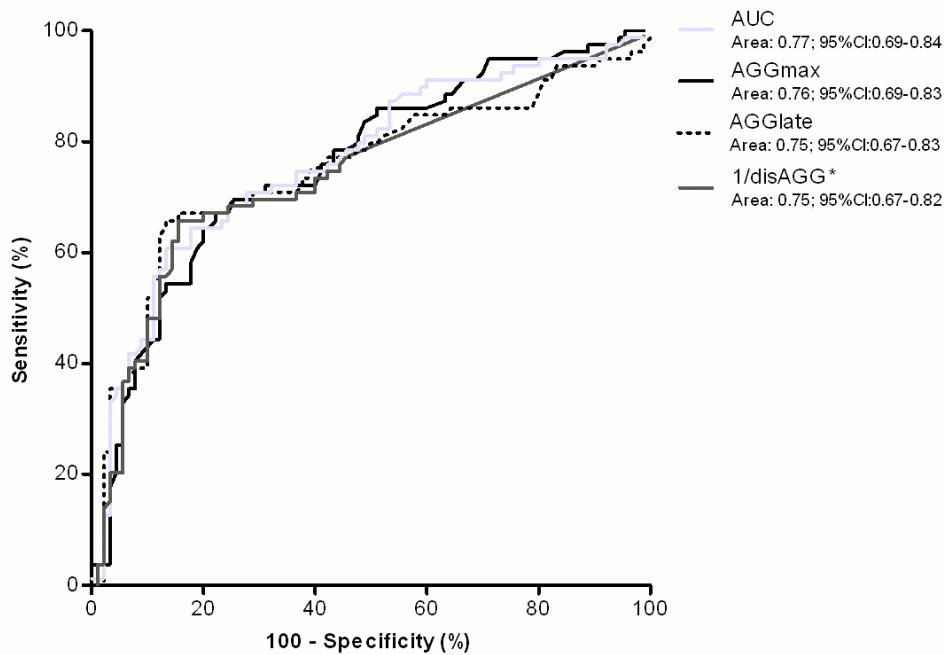


Figure 11. Receiver-operator characteristic (ROC) curve analysis of the ability of light transmission aggregation values to predict high platelet reactivity defined by VASP-PRI>50%. The area under the ROC curve is shown with the 95% confidence intervals. AUC: area under the light transmission curve; Agg<sub>max</sub>: maximal aggregation; Agg<sub>late</sub>: 6-minute late aggregation; disAGG: disaggregation.

\*1/disAGG: as disaggregation is inversely correlated to VASP, its reciprocal was used for better comparability with other parameters.

## Discussion

Our results confirm that Agg<sub>max</sub> values are significantly influenced by both the P2Y1 and P2Y12 receptors, while 6-minute Agg<sub>late</sub> values are less dependent from P2Y1-activity. When LTA estimates were compared to VASP-PRI we registered significant, moderate-strength relationship without substantial differences between the tested parameters. The significant correlation validates LTA assessment for monitoring the efficacy of thienopyridine therapy

and is in line with prior studies that had compared VASP with LTA. (20) Our study is the first to compare several estimates of LTA to VASP-PRI in a prospectively recruited, homogenous stable angina patient population. Based on the results, the recorded LTA estimates were similar in correlation to VASP-PRI without the superiority of  $Agg_{late}$  over  $Agg_{max}$ , AUC or disAGG in monitoring the degree of P2Y12-receptor inhibition. According to the highest correlation coefficient and the result of the multivariable linear regression analysis, not  $Agg_{late}$ , but AUC seems to be the best linear predictor of VASP-PRI. These results might be partly surprising as the in vitro tests confirmed that  $Agg_{max}$  was greatly influenced by the P2Y1-receptor activation, while  $Agg_{late}$  was less dependent. However, only a small proportion of patients had high  $Agg_{max}$  yet low VASP-PRI values, suggesting that the overestimation of P2Y12 receptor inhibition by  $Agg_{max}$  cannot be confirmed. Contrary, all the LTA estimates frequently failed to detect an impaired P2Y12 receptor inhibition. This feature was also recently emphasized by Schäfer et al. and might be explained with the differences in the methodologies. (57)

Bland-Altman plots were useful to confirm that in spite of the significant correlation there might be substantial differences in certain individuals between VASP and LTA-defined platelet reactivity. Based on these,  $Agg_{late}$ , disAGG and AUC are systematically underestimating VASP-defined platelet reactivity in the low platelet reactivity range (markedly below 50% platelet reactivity). As differences between VASP-PRI and LTA estimates might reach up to half of the measured parameter, it might be clinically meaningful. Likewise, the two methods of testing the efficacy of thienopyridine therapy are closely related, but not replaceable.

Based on the ROC curves we defined optimal cutoff values for LTA parameters to separate NPR and HPR patients. Due to previous clinical data, a VASP-PRI greater than 50% was considered as HPR and a  $PRI < 50\%$  was defined as normal platelet reactivity. (58) Using the

optimal thresholds to achieve the highest categorical agreement with VASP-defined categories,  $Agg_{late}$  showed the highest categorical agreement. This means that the only benefit of preferring  $Agg_{late}$  is the more precise classification of patients with high or normal platelet reactivity. It is still a matter of debate which is more important: a higher categorical agreement or a better linear correlation with VASP-PRI. This question should be answered based on clinical evidence. If the association between adverse clinical events and the efficacy of P2Y12 receptor inhibition is linear, stronger correlation and better linear agreement is probably more important. Contrary, if the occurrence of thrombo-ischemic complications is threshold-specific, i.e. patients above a prespecified cutoff are prone to develop such events, better agreement with the defined categories is more useful, regardless the possible disagreements in certain levels of the variables. As up to now we are lacking peer data that may clarify this issue, it remains to be answered.

## Conclusion

In conclusion, our results show that all the examined parameters of LTA are in significant association with VASP-PRI without the superiority of  $Agg_{late}$  over the others in linear regression or in predicting high platelet reactivity. Based on the optimal cutoff values of the ROC curve, the only benefit of  $Agg_{late}$  is the more precise classification of patients to VASP-defined normal or high platelet reactivity. The significant association validates LTA for monitoring P2Y12 receptor inhibition; however, there might be clinically meaningful discrepancies in certain individuals between results of the VASP and LTA assessment.

## 5.2 The efficacy of thienopyridine therapy influences late outcome after coronary stent implantation

### Background:

The introduction of ADP receptor blocker thienopyridines for the supplementation of aspirin monotherapy improved the outcome of percutaneous coronary interventions (PCI) by decreasing the occurrence of stent thrombosis. (5-8) At present, antiplatelet agents are used in uniform, fixed-dose manner despite the growing body of evidence supporting large inter-individual variability in response to both aspirin and clopidogrel. (20, 45) Inter-individual differences indicate that a substantial number of patients persist with high platelet reactivity despite aspirin and thienopyridine treatment after coronary intervention. It is still debated whether low response to therapy and consequential high platelet reactivity is associated with adverse thrombotic events. Moreover, no consensus exists on defining the threshold for low response to therapy; however, this threshold should be clinically adjusted.

### Objective:

To evaluate the clinical impact of inter-individual differences in response to antiplatelet therapy measured with LTA after PCI, we designed a prospective study. We hypothesized that a good response to antiplatelet agents and thereby a lower level of platelet reactivity is associated with better long-term clinical outcome after PCI.

## Methods

### Study Population

Patients referred for coronary angiography and scheduled for an ad hoc PCI were recruited. Patients were considered eligible for enrolment in the study if they had clinically proven stable or unstable angina, had de novo lesion in one of the main coronary arteries causing a diameter stenosis over 50%, were feasible to direct stent implantation, were at least 18 years, and provided informed consent before enrolment. Exclusion criteria included contraindications to any antiplatelet/antithrombotic agents, haematological disorders and coagulopathies, 2 or 3 vessel disease with an indication for coronary artery bypass grafting, recent myocardial infarction (within 72 hours), poor left ventricle function (ejection fraction below 25%), history of stroke in the past 6 months and any other known disorder that may significantly influence survival (malignancy, serious liver disease, immunosuppressed conditions).

### Protocol of Percutaneous Coronary Intervention

Ad hoc stent implantations were done using bare metal stents. The procedures were performed from femoral approach after the administration of 60 to 90 U/kilograms of unfractionated heparin. Antiplatelet agents were given in an oral bolus at the time of the procedure, 300 mg clopidogrel or 500 mg ticlopidine with 300 mg aspirin at the discretion of the operator. No platelet glycoprotein IIb/IIIa receptor blocker was used. After PCI, 75 mg clopidogrel or 2x250 mg ticlopidine were administered with 100 mg enteric-coated aspirin therapy until 12 months.

### Platelet Function Analysis

Platelet aggregation studies were performed  $30 \pm 5$  days after the intervention with LTA (see section 4.1). Efficacy of thienopyridine therapy was measured with ADP 5 and 10  $\mu$ M, while

efficacy of aspirin treatment was evaluated with collagen 2 µg/mL and adrenaline 10 µM. At the same time, 5 ml blood was drawn for assessment of plasma concentration of soluble markers of platelet activation including soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble CD40 ligand (sCD40L) and soluble P-selectin (sP-selectin) using Human Cardiovascular Multiplex Fluorescent Bead Immunoassay (Bender MedSystems GmbH, Vienna, Austria, see section 4.3). Detailed descriptions of the analyses are described above.

## Outcome

The primary composite end point of the study was the occurrence of cardiovascular death or MI or revascularization at 10 months. Revascularizations included repeated PCI and CABG. Repeat coronary angiographies were indicated by clinical signs or diagnostic methods, showing recurrent myocardial ischemia. Early postprocedural adverse events occurring within 30 days of the follow-up included cardiovascular death, MI, or urgent target-vessel revascularization. All potential adverse events were screened during regular visits 30 and 300 days after the initial intervention that included physical examination and resting ECG.

## Statistical Analysis

All analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, Illinois). Univariate Cox proportional hazard models were used to evaluate the relation of plasma level of activation markers and maximal aggregation values to primary outcome. Cumulative event-free survivals in study groups were compared with the Kaplan-Meier-test. P values smaller than 0.05 were considered statistically significant

## Results

Between May 2003 and January 2005, 134 patients satisfied our entry criteria and were enrolled. After the intended follow-up period of 10 months, 33 major adverse cardiac events (MACE) were traced (Table 3). Adverse events developed  $151 \pm 111$  days after the index intervention, with 2 end points occurring within the first one month. These patients developed subacute stent thrombosis on day 7 and 13. No cardiac or non-cardiac deaths were recorded, and 33 revascularizations were performed, including 10 CABG and 23 repeated PCI. We registered 3 cases of MI that were revascularized with primary PCI. Two of them were due to subacute stent thrombosis, whereas one de novo lesion triggered an athero-thrombotic event. On the basis of the control coronary angiographies, we found 27 cases of significant instent restenosis (ISR).

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Table 3. Major adverse cardiac events (MACE) at 10 months in 134 patients after PCI

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Death	0 (0)
Myocardial Infarction	3 (2.2)
Revascularisation	33 (24.6)
Revascularisation with PCI	23
Revascularisation with CABG	10
Death or Myocardial infarction or revascularisation (1 <sup>o</sup> endpoint)	33 (24.6)
Instent Restenosis	27 (20.1)
Stent Thrombosis	2 (1.5)
De Novo Lesion	5 (3.7)

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According to results of LTA assessments,  $\text{Agg}_{\text{max}}$  values were  $33 \pm 14.83$  with ADP,  $25 \pm 15.60$  using adrenaline and  $20 \pm 22.96$  in case of collagen stimuli. The distribution of ADP-induced maximal aggregation values has shown a Gaussian pattern, whereas efficacy of aspirin



showed a detached group of nonresponders delineated. (Figure 12) Maximal platelet aggregation values to ADP 5 and 10  $\mu$ M were in significant relation with MACE in the Cox regression model ( $p < 0.01$  in both cases), indicating better survival in case of more effective inhibition. In Kaplan-Meier analysis, cumulative event-free survival of patients in the lower 50 percentile of ADP-induced platelet aggregation was significantly better than that of patients in the upper 50 percentile. (Figure 13A) Overall, patients in the higher 50% of ADP-aggregation had a 6.84-fold HR for MACE (95%CI: 2.64-17.72,  $p < 0.001$ ).

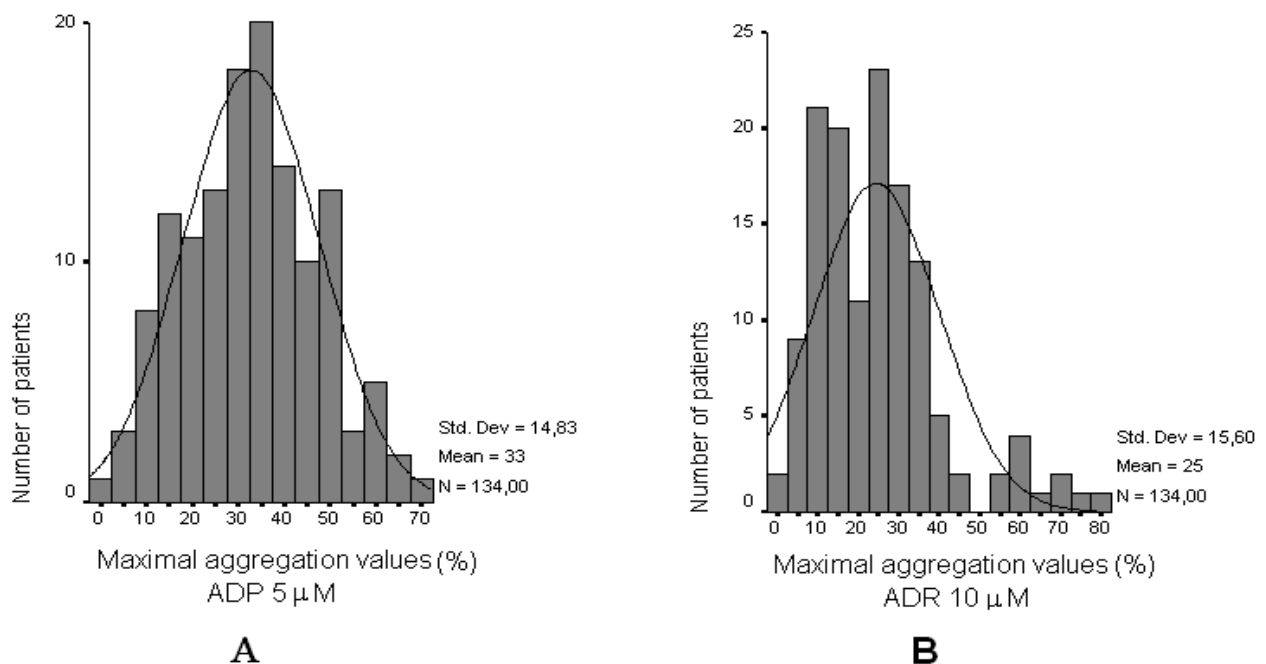


Figure 12. Distribution of maximal platelet aggregation ( $Agg_{max}$ ) values using adenosine diphosphate (ADP) 5  $\mu$ M (Panel A) and adrenaline (ADR) 10  $\mu$ M (Panel B) reflecting the efficacy of thienopyridine and aspirin therapy, respectively. Response to thienopyridine therapy shows a normal distribution pattern (Panel A), while in case of aspirin patients with decreased efficacy constitute a segregated group above 50% of  $Agg_{max}$  values.

In case of collagen 2  $\mu\text{g}/\text{mL}$  and adrenaline 10  $\mu\text{M}$ , no association was detected with primary outcome ( $p=0.84$  and  $p=0.76$ , respectively). However, the plasma level of soluble P-selectin proved to be an independent risk factor of the MACE ( $p<0.05$ ) as lower levels were associated with better outcome. This benefit is demonstrated in the Kaplan-Meier diagram as well, where event-free survival of the lower 50-percentile group is significantly better (Figure 13B). Neither sVCAM-1 nor sCD40L had association with MACE in the Cox regression analysis.

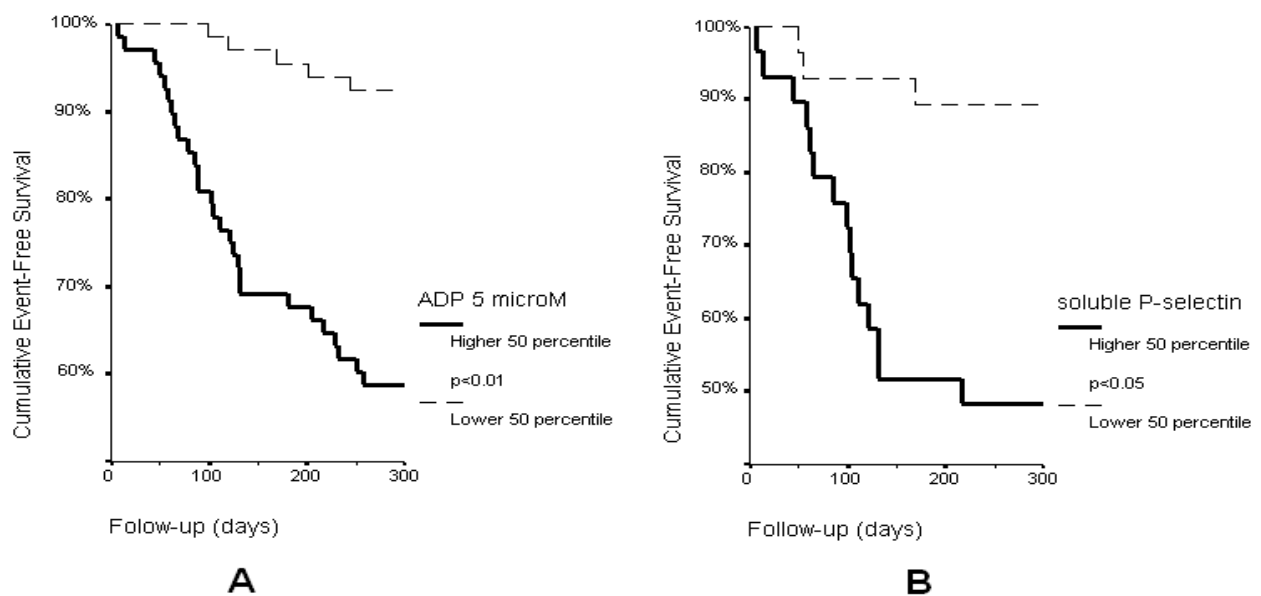


Figure 13. Kaplan-Meier diagrams of the cumulative event-free survival of patients after coronary stent implantation. Panel A: lower and higher 50 percentiles of maximal platelet aggregation values using ADP 5 $\mu\text{M}$  ( $p<0.01$ ). Panel B: lower and higher 50 percentiles of plasma level of soluble P-selectin ( $p<0.05$ ).

## Discussion

The major finding of this study is that LTA-monitored efficacy of thienopyridine therapy is in significant relation with the occurrence of major adverse cardiac events after PCI, showing clinical benefit in patients at more effective platelet inhibition. As sP-selectin is a sensitive marker of platelet activation, the relation between plasma levels and MACE supports independently that the degree of platelet activation may interfere with clinical outcome. Notably, the efficacy of aspirin therapy assessed with collagen and adrenalin had no association with the primary endpoint.

These results are in line with previous observational studies suggesting a significant link between the efficacy of LTA-measured ADP-receptor inhibition and recurrent thrombotic events in patients after PCI. The study of Matetzky et al. was the first to support that acute coronary syndrome patients in the highest quartile of ADP-induced platelet aggregation had a higher risk to MI, stroke, and peripheral atherothrombosis in one month after PCI. (49) Cuisset et al. demonstrated that NSTEMI patients with high post-treatment platelet aggregation had significantly higher risk to 30-day cardiovascular events and stent thrombosis. (51) Our results corroborate that ADP-induced platelet aggregation values measured at maintenance phase of dual antiplatelet therapy after PCI are associated with long-term outcome. Comparison of patients with different response to ADP-induced platelet aggregation revealed that not just a minority, but 50% of patients with high platelet reactivity had higher risk to 10-month MACE. As the median value separated patients in terms of outcome, it might be a clinically relevant threshold for defining low response to clopidogrel therapy.

Notably, the composite end point of death, MI, and repeat revascularization was dominantly triggered by ISR rather than thrombotic events. According to the control coronary angiographies, 27 patients were found to have significant ISR in the study stent. As 82% (27 out of 33) of the primary end point was triggered by ISR, some link between the thrombocyte

reactivity and ISR should be hypothesized. These results suggest that the more effective the thienopyridine therapy, the less frequent the need for repeat revascularization. As neither the design, nor the sample size of the study was powered to evaluate this hypothesis, further research is needed to clarify the link between high platelet reactivity and ISR.

## Conclusion

Interindividual variability in efficacy of thienopyridine therapy has substantial clinical importance. While efficacy of aspirin therapy did not interfere with 10-month occurrence of death, MI or revascularisation, patients in the lower 50% of ADP-induced platelet aggregation had better long-term outcome after PCI.

### 5.3 The impact of diabetes mellitus on the efficacy of combined antiplatelet therapy after coronary stent implantation

#### Background

Aspirin plus thienopyridine therapy is one of the foot-stones of clinical success after coronary stent implantation. (18, 19) However, impaired response to aspirin and/or thienopyridines has been demonstrated and may contribute to the development of major adverse cardiac events (MACE). In a previous study, we demonstrated that patients above the median value of LTA-assessed  $Agg_{max}$  had a significantly higher risk for death, MI or revascularization after PCI. (59) Thus, high post-treatment platelet reactivity is a major predictor of worse clinical outcome after PCI. Several factors might contribute to the inappropriate response to clopidogrel: genetic polymorphisms involving the absorption capacity or the activity of the CYP450 enzyme system; clinical conditions such as acute coronary syndrome and low patient compliance. (60) Some of these features can easily be assessed enabling a risk prediction for the estimated antiplatelet response. (61)

#### Objectives

In this examination, we sought to evaluate the impact of type two diabetes mellitus (DM) on the efficacy of antiplatelet therapy in patients after coronary stent implantation.

#### Methods

With a retrospective search in our LTA database in patients after PCI, we collected diabetic (DM) and matching, (according to age, gender, risk profile, stent type and antiplatelet regimen) non-diabetic (ND) control subjects in order to compare their efficacy of antiplatelet therapy.

Among diabetics, we differentiated oral antidiabetic- (OAD) and insulin-treated (INS) groups in whom fasting plasma glucose was also measured after stent implantation.

Blood was drawn for LTA assessment >6 hours of PCI, after receiving a 600-mg loading dose of clopidogrel and 300 mg aspirin. 1x75 mg clopidogrel and 1x100 mg aspirin was administered in the maintenance phase of treatment. ADP 5 and 10  $\mu$ M was used to refer for efficacy of thienopyridine therapy, while collagen 2  $\mu$ g/ml and adrenaline 10  $\mu$ M stimuli for aspirin treatment. LTA assessment was done as described above (Section 4.1). The primary endpoint of the study was the difference in  $Agg_{max}$  values between ND and DM groups. Secondary analyses focused on  $Agg_{max}$  values in diabetic subgroups and correlation of actual fasting glucose to  $Agg_{max}$ .

Table 4. Clinical characteristics in diabetic (DM) and non-diabetic (nonDM) patients

	DM (n=79)	nonDM (n=81)	p Value
Male, n(%)	45(57)	51(63)	Ns
Age, mean $\pm$ SD (ys)	61.83 $\pm$ 9.23	59.86 $\pm$ 10.62	Ns
Hypertension, n(%)	67(85)	63(78)	Ns
Smoking/currently, n(%)	17(22)/6(8)	22(27)/9(11)	Ns
Dyslipidaemia, n(%)	63(80)	59(73)	Ns
ACS/stable angina, n(%)	25(32)/54(68)	31(38)/50(62)	Ns
Prior MI, n(%)	23 (30)	20(25)	Ns
Prior PCI, n(%)	28 (35)	25(31)	Ns
Prior CABG, n(%)	9 (11)	7(9)	Ns
BMS/DES/POBA, n(%)	62(79)/15(20)/2(1)	69(85)/9(11)/3(4)	Ns
Days of LTA after PCI, n(%) (0-1 /2-7 / $\geq$ 7)	28(35)/17(22)/34(43)	33(41)/10(12)/38(47)	Ns

## Results

We found 79 type II. diabetic patients for evaluation in the database and matched them with 81 control subjects. Clinical characteristics between diabetic and non-diabetic groups are presented in Table 4. According to these, no significant differences were observed. Among the 79 DM patients, 56 (71%) were on oral glucose-lowering therapy and 23 (29%) received insulin treatment.

On the basis of the LTA measurements, ADP-induced  $\text{Agg}_{\text{max}}$  values did not differ significantly between the DM and ND group (ADP 5  $\mu\text{M}$ :  $P=0.32$ ; ADP 10  $\mu\text{M}$ :  $P=0.47$ ). In diabetic patients,  $\text{Agg}_{\text{max}}$  values of the OAD group were lower than that of the INS group, however, the difference did not reach the level of significance (ADP 5  $\mu\text{M}$ :  $P=0.07$ ; ADP 10  $\mu\text{M}$ :  $P=0.06$ ). Meanwhile, efficacy of thienopyridine therapy showed significant difference between INS and ND patients (ADP 5  $\mu\text{M}$  and ADP 10  $\mu\text{M}$ :  $p<0.05$ ). (Figure 14A, C and E) Using our previous results for the definition of high platelet reactivity (HPR:  $\text{Agg}_{\text{max}}>33\%$ ), insulin-treated patients had an OR of 2.46 (95%CI: 0.95-6.36,  $p<0.05$ ) for HPR compared to non-diabetics. (59)

We found no relevant differences in efficacy of aspirin therapy in group comparisons according to adrenalin and collagen-stimulated measurements. (Figure 14B, D and F) Notably, the level of fasting glucose did not correlate with  $\text{Agg}_{\text{max}}$  values (Spearman  $r=0.13$ ,  $p=0.32$ ).

## Discussion

In this study, we demonstrated that patients with insulin-treated DM have impaired response to clopidogrel. Insulin has specific effects on platelets, as insulin receptor complex is expressed on their surface. Insulin mediates inhibition of platelet activation through inactivating the  $\text{G}_{i\alpha}$  protein that leads to an increase in cAMP formation in the platelet. Rise in cyclic AMP levels results in phosphorylation of intracellular proteins that prohibit platelet

activation. In diabetic subjects, insulin-resistance has a major role in the development of multi-organ damage. Ferreira et. al demonstrated that antiplatelet effects of insulin are diminished in diabetic patients that leads to higher platelet reactivity. (62) Moreover, endothelial dysfunction with lower prostacyclin production might also contribute to HPR in diabetics. As both insulin and ADP-receptor converge to  $G_{i\alpha}$ -protein, diminishment of  $G_i$  inhibition in diabetics might explain the excess activity of ADP-receptor, with consequently lower response to clopidogrel. (63)

## Conclusion

Efficacy of thienopyridine therapy is impaired among insulin-treated patients compared to non-diabetic ones after PCI. Fasting glucose level after PCI was not associated with platelet reactivity.



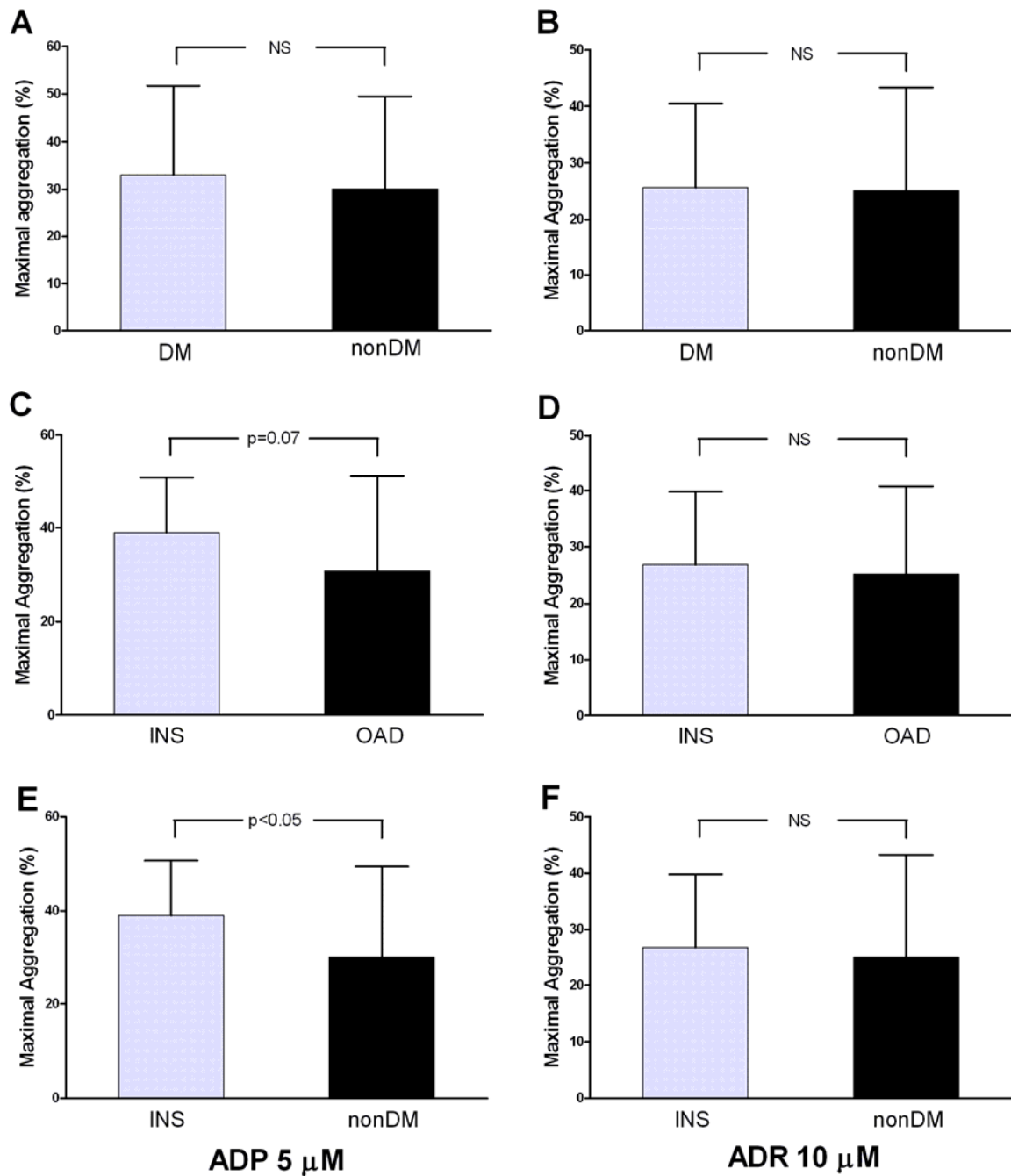


Figure 14. Inter-group comparisons in maximal aggregation values reflecting the efficacy of clopidogrel (Panel A, C, E using ADP 5  $\mu$ M) and aspirin therapy (Panel B, D, F using adrenalin 10  $\mu$ M). DM: type II. diabetes mellitus, nonDM: non-diabetic patients, INS: insulin-treated diabetes mellitus, OAD: oral glucose-lowering therapy.

## 5.4 Low platelet disaggregation predicts poor response to 150 mg clopidogrel in patients with elevated platelet reactivity

### Introduction

The response to a fixed-dose clopidogrel is not uniform between patients. Growing body of evidence suggests that low response to clopidogrel and consequential high platelet reactivity (HPPR) is associated with adverse thrombo-ischemic events. A meta-analysis of 25 platelet function studies comprising 3688 patients concluded that low response to clopidogrel and consequential high platelet reactivity is an independent predictor of ischemic cardiovascular events and subacute stent thrombosis. (54) Similarly, in a prospective cohort of 134 patients we found that patients with  $\text{Agg}_{\text{max}}$  values  $>33\%$  had a 6.84-fold HR for MACE (95CI:2.64-17.72,  $p<0.001$ ) compared to those with  $\leq 33\%$ . (59) Recently, numerous antiplatelet protocols were tested in order to overcome elevated platelet reactivity. (64-69) Doubling the maintenance dose of clopidogrel after PCI is a promising option and has been recommended in patients in whom less than 50% of platelet inhibition is demonstrated. (18) Importantly, all of the studies that evaluated the biological effect of 150 mg clopidogrel described large inter-individual differences in the extent of the benefit, indicating that there are patients who profited from dose-shift while others persisted with EPR. As the latter might remain at higher risk for adverse outcome, identification of variables that predict the response to 150 mg clopidogrel is awaited.

### Objective

The main aim of this study was to investigate pharmacological benefits of administering a high maintenance dose (150 mg) of clopidogrel as compared to standard therapy (75 mg) in

patients with verified HPPR after 600-mg loading dose. Furthermore, we sought to analyze possible determinants of response to 150 mg clopidogrel.

## Methods

### Patients and study design

We aimed to recruit three patient populations to compare their efficacy of clopidogrel treatment at two separate time points after PCI: a group of good responders receiving 75 mg clopidogrel (no HPPR), a group of patients with HPPR taking 75 mg (HPPR+75 mg) and a group of HPPR subjects with increased maintenance dose of clopidogrel (HPPR+150 mg). A detailed flow-chart of the study design is depicted in Table 5. Clopidogrel-naïve patients who underwent PCI with stent implantation after the administration of 600 mg loading dose (LD) of clopidogrel were eligible. Both stable angina and acute coronary syndrome patients were enrolled. Exclusion criteria included recent (<7 days) treatment with thienopyridines, administration of GPIIb/IIIa inhibitors, concomitant anticoagulant therapy, recent (<6 months) hemorrhagic stroke, intolerance to clopidogrel/aspirin and low platelet count ( $<100 \times 10^9/L$ ).

In 2006, we measured the efficacy of antiplatelet therapy in patients after PCI without adjusting the maintenance dose of clopidogrel in low responders. Following retrospective analysis of the database, 1671 patients underwent PCI during this period in the Heart Institute, University of Pécs. Due to predefined exclusion criteria, 1174 patients were excluded. Among 497 eligible, 235 patients had LTA assessments at the two predefined time points: at 6-24 hours and at 15 to 30 days after PCI. From these patients, 85 were identified with HPPR who received 75 mg clopidogrel for maintenance-phase therapy. These patients formed the retrospective, per-protocol group of HPPR+75 mg patients.

Into the interventional arm of the study, we aimed to prospectively recruit 85 HPPR patients to have a 1:1 ratio of HPPR subjects for the comparison. These patients received 150 mg

clopidogrel for 30 days after PCI (HPPR+150 mg). From January 2007, 202 patients were screened to have 85 HPPR patients. Likewise, 117 consecutive patients with good response to clopidogrel formed the no HPPR group who received standard 75 mg clopidogrel. The study protocol was approved by the Regional Ethics Committee (Approval No.:2870), and patients gave written consent for participation.

#### Blood sampling and aggregometry

All patients received a 600-mg LD of clopidogrel and 300 mg aspirin immediately after coronary angiography showing suitable lesions for ad hoc stent implantation. 6 to 24 hours after the administration of LD (T1), and at 15 to 30 days after PCI (T2), 10 ml blood was drawn for LTA measurement and the assessment was performed as described in section 4.1.

#### End points and sample size calculation

The primary end point of the study was 5  $\mu$ M-induced  $Agg_{max}$  measured at study time point 2 (T2) between HPPR groups. Secondary end points included estimates of  $Agg_{max}$ ,  $Agg_{late}$  and  $disAgg$  with inter- and intragroup comparisons, and the relative percentage of HPPR patients in each group at T2. We aimed to determine the independent clinical, procedural and platelet aggregation predictors of response to 150 mg maintenance dose of clopidogrel. According to sample size calculation, we assumed that choosing a power of 95% and a two-sided  $\alpha$ -level of 0.05, at least 25 patients in each group were required to detect a 15% reduction in mean  $Agg_{max}$  values (from  $50\pm 15\%$  to  $42.5\pm 15\%$ ) at T2 in the HPPR + 150 mg clopidogrel group compared to that of HPPR+75 mg patients (64).

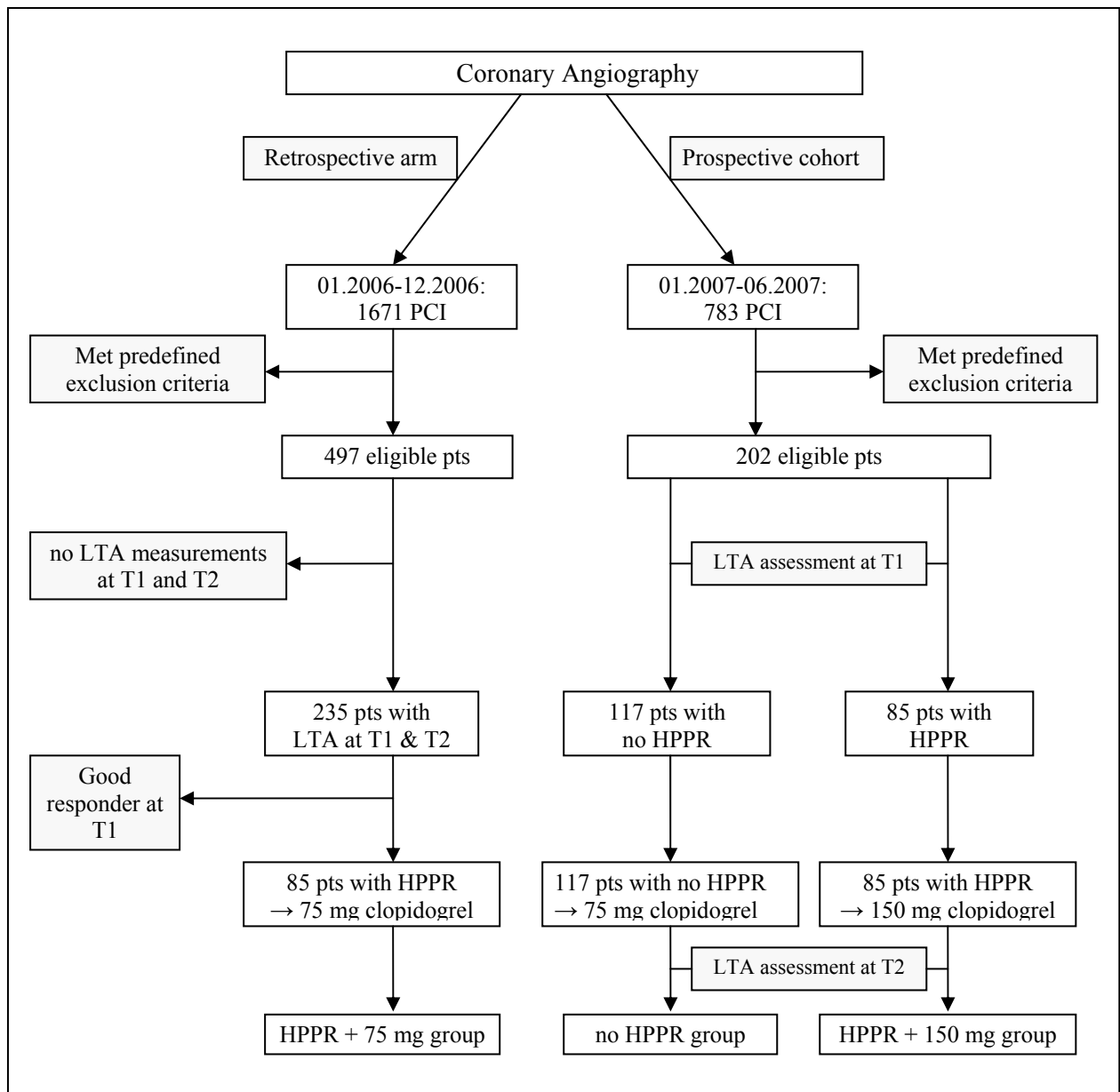


Table 5. Flow chart of study design. PCI: percutaneous coronary intervention; pts: patients; LTA: light transmission aggregometry; HPPR: High post-treatment platelet reactivity; T1: time point 1; T2: time point 2.

## Results

### Patient characteristics

The study cohort comprised 287 patients after PCI. 85 patients with verified baseline HPPR received 150 mg maintenance dose until 30 days of PCI (HPPR + 150 mg), 85 patients with

HPPR were administered the standard 75 mg clopidogrel (HPPR + 75 mg) and 117 good responder ones (no HPPR) received 75 mg clopidogrel daily after coronary stent implantation.

TABLE 6. Baseline Clinical and Procedural Characteristics in HPPR Groups

	HPPR + 75 mg n=85	HPPR+150 mg n=85	p Value
Age, mean $\pm$ SD(ys)	61.3 $\pm$ 11.1	62.2 $\pm$ 10.2	NS
Male gender, n(%)	49(57.6)	53(62.4)	NS
Active smokers, n(%)	20(23.5)	17(20.0)	NS
Arterial hypertension, n(%)	63(74.1)	59(69.4)	NS
Diabetes mellitus, n(%)	20(23.5)	16(18.8)	NS
Dyslipidaemia, n(%)	66(77.6)	59(70.2)	NS
Low cardiac output (EF<40%) n(%)	18(21.1)	16(18.8)	NS
Prior myocardial infarction, n(%)	16(18.8)	20(23.5)	NS
Prior stroke, n(%)	6(7.1)	5(5.9)	NS
Prior PCI, n(%)	23(27.1)	24(28.2)	NS
Prior CABG, n(%)	16(18.8)	14(16.5)	NS
Stable angina, n(%)	43(50.6)	46(54.8)	NS
Acute coronary syndrome, n(%)	42(49.4)	38(45.2)	NS
Vessel treated, n(%)			NS
LM	5(5.9)	3(3.5)	NS
LAD	41(48.2)	40(47.1)	NS
Cx	17(20.0)	21(24.7)	NS
RCA	36(42.4)	33(38.8)	NS
VSG	3(3.5)	8(9.4)	NS
Stent type, n(%)			NS
DES	27(31.8)	31(36.5)	NS
BMS	58(68.2)	53(62.4)	NS
Aspirin, n(%)	79(92.9)	82(96.5)	NS
Beta-blocker, n(%)	70(82.4)	71(83.5)	NS
CYP3A4-metabolized statin, n(%)	59(69.4)	56(65.9)	NS
non-CYP3A4 metabolized statin, n(%)	16(18.8)	21(24.7)	NS
ACEI or ARB, n(%)	62(72.9)	73(85.9)	NS
Proton pump inhibitor, n(%)	20(23.5)	23(27.1)	NS

HPPR: high post-treatment platelet reactivity; EF: ejection fraction; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; VSG: vena saphena graft; DES: drug-eluting stent; BMS: bare metal stent; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin II receptor blocker.

As the primary endpoint of the study was comparison of  $Agg_{max}$  values at T2 in groups of HPPR, we compared baseline clinical and procedural characteristics with medication profile of in these patients. (Table 6) Based on these, no significant differences were revealed.

According to personal visit at study time point 2, all patients were compliant to the prescribed medication and no patients interrupted clopidogrel or aspirin therapy arbitrary or as a result of side effects. According to Thrombolysis In Myocardial Infarction criteria, two minor bleeding events occurred in the HPPR+150 mg group between day 1 and 30. No major bleeding events were registered. During the 30-day study period, 2(2.4%) subacute stent thromboses occurred in the HPPR+150 group while 3(3.5%) were registered in HPPR+75 mg patients. Good responders to clopidogrel (no HPPR) incurred no adverse ischemic events during 30 days.

### Platelet aggregation profiles

Maximal aggregation, late aggregation and disaggregation values  $\pm$ SD are presented and compared in Figure 15 and 16. At baseline, after the loading dose of 600 mg clopidogrel no significant differences were observed in  $Agg_{max}$  between groups of HPPR. At maintenance phase, all three patient groups showed significant changes in  $Agg_{max}$  values compared to baseline indicating differences in antiplatelet efficacy between the loading phase and the maintenance period. Administering 150 mg clopidogrel resulted in significantly lower  $Agg_{max}$  at time point 2 compared to 75 mg in patients with high post-treatment platelet reactivity supporting the enhanced P2Y<sub>12</sub> receptor inhibition due to higher dose of clopidogrel. (Figure 15A, 16A) In parallel, the relative percentage of HPPR patients decreased significantly with the high maintenance dose. (Figure 15B) However, despite administering 150 mg clopidogrel neither  $Agg_{max}$  nor the prevalence of HPPR patients reached that of good responders. (Figure 15, 16A)

$Agg_{late}$  values have changed consistently with  $Agg_{max}$  in patient groups. (Figure 16B) With no significant differences at baseline in both groups with HPPR, 150 mg clopidogrel resulted in a significant decrease in  $Agg_{late}$  compared to 75 mg maintenance therapy. However,  $Agg_{late}$  of good responder patients was still lower than that of patients on 150 mg clopidogrel.

Platelet disaggregation, reflecting the reversibility of the aggregation curve (Figure 17) was significantly higher in good responders compared to HPPR patients both at baseline and at maintenance phase. With no differences at baseline, 150 mg clopidogrel increased disAgg significantly compared to 75 mg. (Figure 16C)

### Determinants of response to 150 mg maintenance dose

To determine variables that significantly correlate to  $Agg_{max}$  at T2, linear regression models were generated including estimates of the aggregation curve ( $Agg_{max}$ ,  $Agg_{late}$ , disAgg, Figure 3, 4) as well as clinical/procedural variables. In univariate linear regression models, baseline  $Agg_{max}$ ,  $Agg_{late}$ , disAgg, administration of beta-blockers and CYP3A4-metabolized statins as well as acute coronary syndrome correlated significantly with primary endpoint. Active smoking, drug-eluting stent implantation and proton pump inhibitors also showed a trend ( $p < 0.10$ ) towards. However, in multiple linear regression analysis, disaggregation and acute coronary syndrome prevailed as the independent predictors of maintenance-phase  $Agg_{max}$  values. To corroborate these findings, multivariate logistic regression models were used to calculate odds ratios for HPPR. Without adjustment for confounding clinical and procedural variables, disaggregation (per 1% increase) and acute coronary syndrome (ACS) were associated with an OR for HPPR of 0.95 (95% CI: 0.92-0.98,  $p=0.002$ ) and 5.82 (95% CI: 1.94-17.47,  $p=0.002$ ), respectively. Adjustment for beta-blockers, CYP3A4-metabolized statins and baseline  $Agg_{max}$  resulted in an adjusted OR of 0.96 (95% CI: 0.93-0.99,  $p=0.009$ ) and 4.83 (95% CI: 1.54-15.09,  $p=0.008$ ) for disaggregation and ACS, respectively. Likewise,



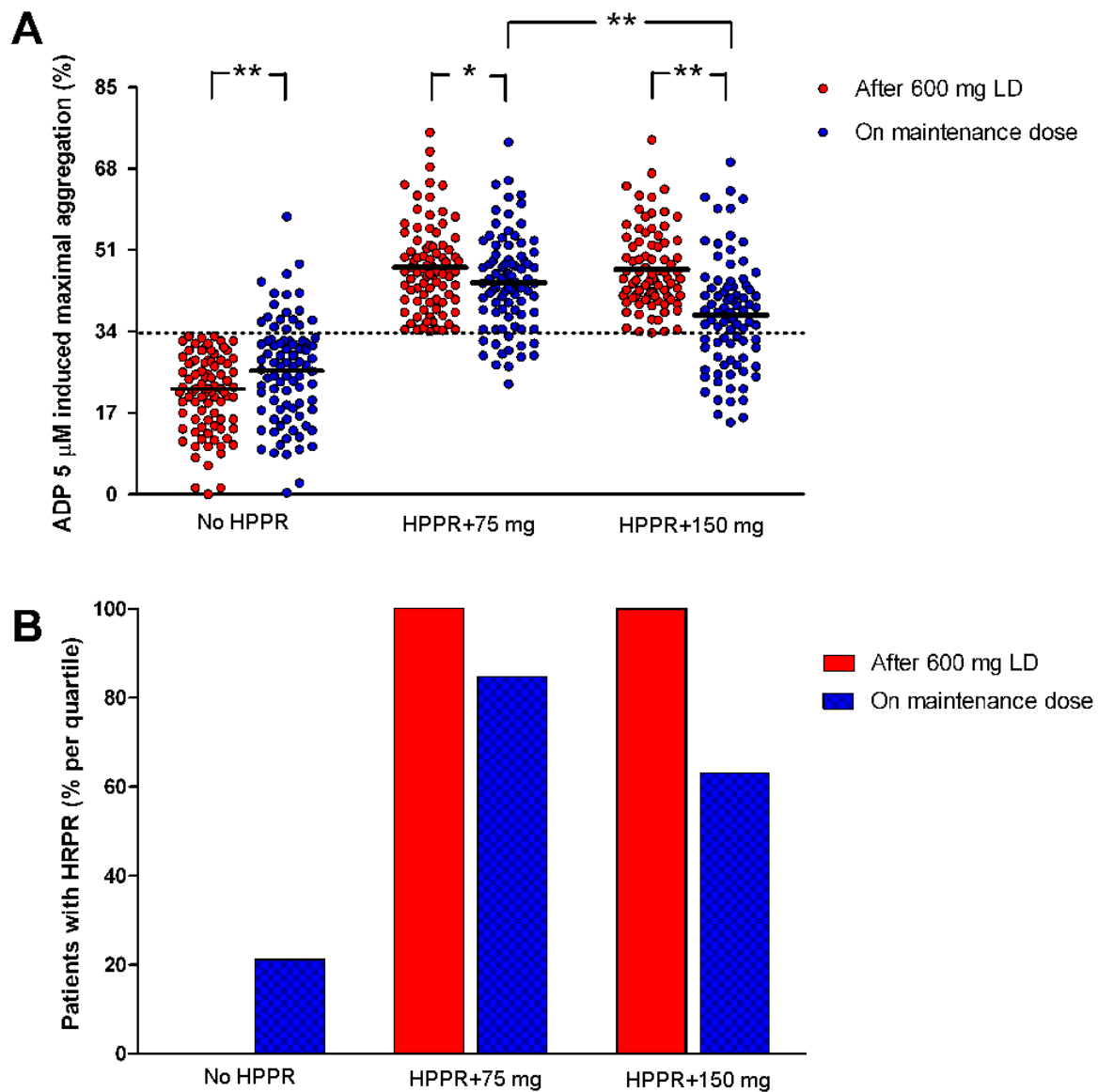


Figure 15. Comparison of platelet function profiles at baseline and on maintenance phase assessed with light transmission aggregometer in patients with good response to clopidogrel (no HPPR), in patients with high post-clopidogrel platelet reactivity receiving 75 mg maintenance dose (HPPR + 75 mg) and in patients with high post-clopidogrel platelet reactivity taking 150 mg clopidogrel (HPPR + 150 mg). Panel A shows group comparison according to  $Agg_{max}$  where dots represent individual data along with the median in each group. The dotted line indicates the cutoff limit for HPPR. (\*:  $p < 0.01$ ; \*\*:  $p < 0.001$ ; LD: loading dose).

Panel B presents the relative percentage of patients with high residual platelet reactivity (HPPR) in study groups between time points.

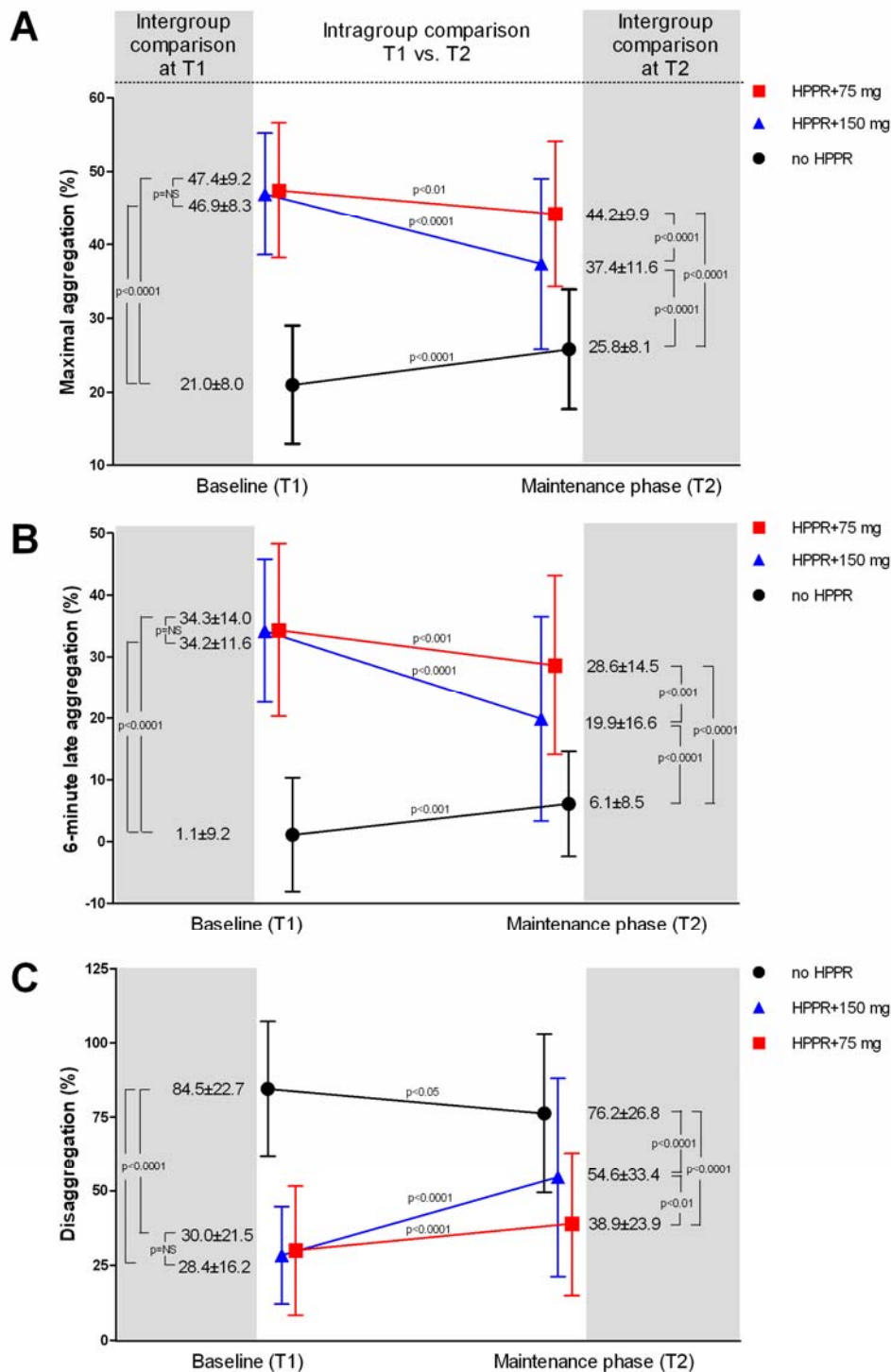


Figure 16. Platelet function profiles at baseline and on maintenance phase of therapy. Maximal aggregation, 6-minute late aggregation and disaggregation with intra- and intergroup comparisons are presented between time points. Mean values (objects)  $\pm$ SD (error bars) are presented at both time points. HPPR: high post-treatment platelet reactivity.

ACS was associated with a 1.7-fold absolute risk of remaining HPPR compared to stable angina following 150 mg clopidogrel. (Table 7)

As the results confirmed that platelet disaggregation predicts the response to 150 mg clopidogrel, the optimal cut-off for predicting normal platelet reactivity ( $Agg_{max} < 34\%$ ) after 150 mg clopidogrel was determined with receiver-operator characteristic (ROC) curve analysis. An area under the curve of  $0.724 \pm 0.055$  (Asymptotic significance: 0.001; 95% Asymptotic Confidence Interval: 0.616-0.833) was obtained and an optimal cutoff value of 16.5% was suggested, with a sensitivity of 94% and a specificity of 43%. Based on these, in HPPR+150 mg group 25 (29.8%) patients were below and 59 (70.2%) above this threshold. Among 25 patients below this cut-off, only 2 patients (8%) achieved a  $Agg_{max}$  value lower than 34% after 150 mg clopidogrel, representing a 92% negative predictive value. Among 59 patients with a disaggregation value higher than 16.5%, 29 (49.2%) showed good response and turned to normal platelet reactivity.

TABLE 7. Predictors of High Post-Treatment Platelet Reactivity at Maintenance Phase with 150 mg Clopidogrel

	Odds Ratio (95% Confidence Interval)	p Value
<b>Univariate analysis</b>		
disaggregation (disAgg)	0.95 (0.92-0.98)	0.002
6-minute late aggregation ( $Agg_{late}$ )	1.08 (1.03-1.14)	0.001
maximal aggregation ( $Agg_{max}$ )	1.08 (1.01-1.16)	0.017
acute coronary syndrome	5.82 (1.94-17.47)	0.002
Beta-blocker	3.34 (0.98-11.34)	0.053
CYP3A4 metabolized statin	3.19 (0.96-10.56)	0.058
<b>Multivariate analysis*</b>		
disaggregation (disAgg)	0.96 (0.93-0.99)	0.009
acute coronary syndrome	4.83 (1.54-15.09)	0.008

\*variables with  $p < 0.10$  were entered in the multivariate analysis

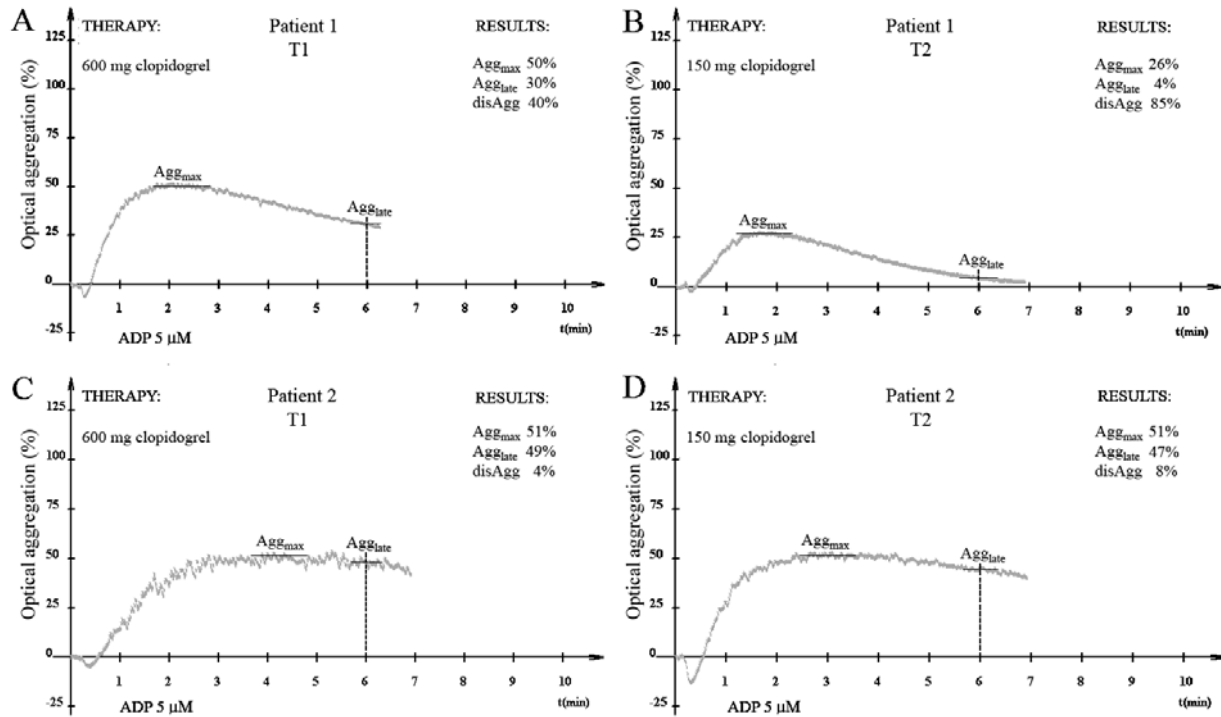


Figure 17. Panels are depicting typical optical aggregation curves after stimuli with ADP 5  $\mu\text{M}$  in two low responder patients with high residual platelet reactivity (HRPR) as assessed with maximal aggregation ( $\text{Agg}_{\text{max}}$ ) values  $\geq 34\%$ . Panel A and C represents the aggregation curve after a 600-mg loading dose (T1) while B and D at maintenance phase (T2) of therapy with 150 mg clopidogrel. In patient 1, 6-minute late aggregation ( $\text{Agg}_{\text{late}}$ ) is 30% with good reversibility indicated by a 40% disaggregation (disAgg) at T1. Administering 150 mg maintenance dose (B) results in a significant decrease in both  $\text{Agg}_{\text{max}}$  (50% vs. 26%) and  $\text{Agg}_{\text{late}}$  (30% vs. 4%) values leading to no HRPR at T2. In patient 2, the aggregation curve shows low disaggregation (4%) indicated by high  $\text{Agg}_{\text{late}}$  value (49%) at T1, and doubling the maintenance dose of clopidogrel has no significant effect on efficacy of ADP receptor inhibition ( $\text{Agg}_{\text{max}}$ : 51% vs. 51%,  $\text{Agg}_{\text{late}}$ : 49% vs. 47%). Disaggregation was defined as  $\text{disAgg}(\%) = (\text{Agg}_{\text{max}} - \text{Agg}_{\text{late}}) / \text{Agg}_{\text{max}} \times 100$ .

## Discussion

Our results confirm that the administration of a high maintenance dose of clopidogrel enhances platelet inhibition and reduces high post-clopidogrel platelet reactivity (HPPR) in a

consecutive cohort of patients after PCI. This impact was characterized by a statistically significant decrease in  $Agg_{max}$  and  $Agg_{late}$  values with increased disaggregation levels among patients receiving 150 mg clopidogrel. These results support the pharmacological benefit of administering 150 mg maintenance dose of clopidogrel in patients with HPPR evaluated after a 600-mg LD. However, the antiplatelet efficacy in response to a 150 mg maintenance dose did not reach the level of patients with normal platelet reactivity. Moreover, less than 40% of the patients with HPPR shifted to normal platelet reactivity. These findings are in line with the results of recent studies investigating the impact of a 150-mg maintenance dose in low responder outpatients. (64-69) The OPTIMUS trial was the first to support the finding that type 2 diabetic patients with LTA-confirmed low response to clopidogrel achieve enhanced platelet inhibition with 150 mg clopidogrel. (64) The study of Fontana et al. also supported the beneficial effects of doubling the maintenance dose in low responder patients using the VASP phosphorylation assay. (65) An important finding of these studies is that administering 150 mg clopidogrel did not reduce inter-individual variability. This means that there are patients who benefit from dose-shift, while in others, HPPR persists despite 150 mg clopidogrel. In a small cohort of low responder patients after a stent thrombosis, Pena et al. evaluated the efficacy of dose titration of clopidogrel in order to override EPR. They concluded that maintenance dose elevation up to 300 mg is time consuming and minimally effective as only two of these patients became responders with the very high doses that also caused side-effects limiting patient compliance. (70) During dose titration, patients with persisting EPR might remain at higher risk to adverse outcome, like the two non-responders in our cohort who suffered stent thrombosis despite the 150 mg maintenance dose. These results suggest that dose elevation to 150 mg is not the right answer in many poor responders to clopidogrel. Likewise, elucidating predictors of response to 150 mg clopidogrel might be of great importance. We evaluated possible clinical, procedural and platelet aggregation

variables in multivariable models and identified platelet disaggregation and acute coronary syndrome as independent predictors of elevated platelet reactivity after 150 mg clopidogrel. Patients with acute coronary syndrome had a 1.7-fold higher absolute risk to persist with EPR compared to those with stable angina. This is concordant with previous studies showing an enhanced platelet activation and aggregation along with an overactive inflammatory cascade in ACS patients, which may consequently attenuate platelet inhibition by thienopyridines. (13) Based on recent studies, new, more potent P2Y<sub>12</sub> receptor antagonists are able to eradicate elevated platelet reactivity and decrease adverse cardiovascular events in ACS patients. (32, 33) Likewise, patients with ACS might be candidates for these alternative antiplatelet strategies.

However, none of these ADP receptor antagonists were tested in stable angina patients after PCI. Moreover, there were more fatal spontaneous bleeding events associated with the use of prasugrel in a non-selected ACS cohort. (32) Likewise, selecting optimal candidates for a higher maintenance dose after an initial platelet function assessment might offer clinically relevant benefits. First, dose adjustment can be selective, administered only for those who show high platelet reactivity, without exposing normal responders to a higher bleeding risk. As bleeding is an independent predictor of poor prognosis after PCI, preventing those events seems equally important as obviating ischemic complications. Secondly, the antiplatelet assessment is not only valuable in selecting patients with HPPR, but to guide the optimal treatment strategy to override poor response. The fact that all the measured platelet function parameters after PCI were significantly associated with the response to 150 mg clopidogrel underscores the importance of these parameters in predicting a response to antiplatelet therapy. Most importantly, our study shows that low platelet disaggregation is the strongest independent predictor of poor response to a high maintenance dose of clopidogrel. High sensitivity at the threshold of 16.5% translated to a 92% negative predictive value in

predicting good response to 150 mg clopidogrel. It is still debated whether platelet disaggregation is an artifact of the ex vivo platelet function assessment or a clinically relevant phenomenon that might occur in vivo as well. A theoretical explanation of the process is that unstable platelet aggregates might fall apart after initial aggregation in continuous magnetic stirring during assessment. Based on our results, patients with a low (16.5%) disaggregation do not show substantial pharmacological benefit from an increased maintenance dose of clopidogrel, while half of those with good disaggregation might shift to normal platelet reactivity.

### Conclusion

In this study, we demonstrate that a 150 mg maintenance dose of clopidogrel is superior to 75 mg in terms of antiplatelet efficacy in patients with elevated platelet reactivity after PCI. However, large inter-individual differences were detected in the response to 150 mg clopidogrel. Acute coronary syndrome and low platelet disaggregation were independent predictors of poor response to 150 mg clopidogrel in the maintenance phase, suggesting that patient selection in the peri-procedural period might predict individual response and help to tailor antiplatelet therapy after PCI.

## 5.5 Transradial versus transfemoral percutaneous coronary intervention in acute myocardial infarction. Systematic overview and meta-analysis

### Introduction

Transradial coronary angioplasty (TRPCI) has gained widespread acceptance since its introduction by Kiemeneij and Laarman. (71) Radial access has been proven to be a highly safe and effective technique for both diagnostic- and therapeutic procedures. (72, 73) Advantages of the transradial approach over the transfemoral include safe and easy haemostasis due to compressibility of the artery, and consequent lack of need for postprocedural bed rest permitting immediate ambulation, greater comfort, and earlier discharge. These have been shown to reduce the costs of hospitalization and improve quality of life for patients. (74, 75) Although it is technically more challenging, transradial intervention is feasible in the setting of acute coronary syndromes. (76-82) The major advantage of the TRPCI is the near elimination of clinically significant access site complications, even in patients at high risk for bleeding (i.e. patients treated with GP IIb/IIIa inhibitors or shortly after systemic thrombolysis). Bleeding events, and the consequent need for transfusion, are independent determinants of survival in acute coronary syndromes. Their relation to short- and long-term mortality has been demonstrated in major randomized trials as well as through the evaluation of registries. (83-86) Thereby, low incidence of vascular access site bleeding complications suggests that the transradial approach may be a safe alternative to the femoral technique employed in acute myocardial infarction with ST segment elevation (STEMI), particularly when an aggressive anticoagulation- and antiplatelet regimen is applied. On the contrary, the possible greater occurrence of procedural failure and longer procedural times occasioned by difficulty in puncturing the radial artery, inability to cannulate the coronaries, or impossibility to perform the angioplasty, are factors that raise concerns as to



whether radial access remains beneficial in the setting where timely reperfusion is critical, in STEMI for instance.

The safety of transradial- and transfemoral PCI in AMI were compared in numerous trials; however, most of them included small patient groups. Despite consistent demonstration of lower bleeding rates, only inconclusive results are available regarding recurrent ischemic events; most of these studies were underpowered to evaluate this issue.

## Objective

Our aim was to perform a systematic review of the literature comparing the safety and efficacy of the two vascular accesses in STEMI and to complete a meta-analysis in order to achieve greater statistical power and more precise effect estimates.

## Methods

### Search strategy

We performed a systematic review of the available literature according to the MOOSE guidelines for the conduct of meta-analyses of observational studies. (87) Relevant studies published between January 1993 and August 2009 were identified from MEDLINE®, SCOPUS®, the Web of Science® with Conference Proceedings, and the Cochrane Central Register of Controlled trials (CENTRAL) using a search strategy that combined text word and MeSH heading. Search keywords included various combinations of the following terms: “transradial”, “radial access”, “myocardial”, “infarct\*“, and “coronary”. No language restrictions were imposed. Furthermore, we searched reference lists of relevant studies and reading reviews and editorials on this topic. In addition, relevant abstracts and presentations from the annual meetings of the American Heart Association, the American College of

Cardiology, the European Society of Cardiology and Transcatheter Cardiovascular Therapeutics were identified.

#### Selection criteria

Inclusion criteria for retrieved studies were a) controlled comparison of the radial- versus femoral approach for coronary intervention b) acute myocardial infarction (either primary- or rescue PCI) and c) intention-to-treat analysis. Exclusion criteria were a lack of clear- and reproducible results and incomplete follow-up, and lack of clear distinction of the clinical setting of the patients included (i.e. separate data for the acute- and elective interventions included).

#### Data abstraction and validity assessment

Data abstraction was independently performed by two unblinded reviewers on pre-specified structure collection forms. Disagreements were resolved by consensus and discussion with a third party. Individual researchers were contacted in the case of incomplete reporting. The quality of study was evaluated by a third investigator according to a score, modified from Jadad et al. and Biondi-Zoccai et al. (72, 88, 89), expressed on an ordinal scale, allocating 1 point for the presence of each of the following: 1) statement of objectives; 2) explicit inclusion and exclusion criteria; 3) description of interventions; 4) objective means of follow-up; 5) description of adverse events; 6) power analysis; 7) description of statistical methods; 8) multicenter design; 9) discussion of withdrawals; and 10) details of medical therapy (e.g., antithrombotic regimens) during- and after coronary procedures. (2)

#### Study outcome measures

The primary clinical outcomes of interest, evaluated at the longest available follow-up, were 1) mortality; 2) major adverse cardiovascular- and cerebrovascular events (MACE), including death, recurrent myocardial infarction, emergency percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG), and stroke; 3) major bleeding (a standardized major bleeding definition was used that was adapted from the meta-analysis of Jolly et al. (73) Briefly, major bleeding was defined as one of the following: fatal bleeding, intracranial hemorrhage, or bleeding associated with a  $\geq 3$  g/dL hemoglobin drop or requiring transfusion or requiring surgery (pseudoaneurysms requiring thrombin injection or ultrasound compression were excluded). For trials where the composite definition was not available, either transfusion rates or proportion of bleeding events associated with a  $\geq 3$  g/dL hemoglobin drop were substituted for major bleeding.)

Secondary procedural outcomes, pooled from individual studies when available, were: procedural time (in minutes), door-to-reperfusion time (in minutes), fluoroscopy time (in minutes), length of hospital stay (in days), and access site crossover.

#### Data analysis and synthesis

Dichotomous variables are reported as proportions and percentages, and continuous variables as mean values and standard deviation. Binary outcomes from individual studies were combined with the Mantel-Haenzel fixed effect model, and the DerSimonian and Laird random effects model was used for sensitivity analysis. Continuous variables were compared with the inverse-variance method to obtain the mean difference and its confidence interval in a fixed-effect method. (90) The odds ratio (OR) and 95% confidence interval (CI) were used as summary statistics for the comparison of dichotomous variables between the radial- and femoral approaches. The mean difference (MD) and 95% CI were used for the continuous variables. Reported values were two-tailed, and hypothesis testing results were considered

statistically significant at p value <0.05. Statistical analysis was performed using the Review Manager 5.0.16 developed and maintained by the Cochrane Collaboration (90). We planned to conduct sensitivity analyses if significant heterogeneity was found for any of the outcomes. Sensitivity- and subgroup analyses were performed using the following categories: 1) randomized and observational studies 2) primary PCI and rescue PCI (studies with >50% of the patients undergoing PCI were included in this group) 3) cohorts whose use of GP IIb/IIIa inhibitor was below and over 15% 4) quality studies whose scores were higher than median and median versus lower than median.

## Results

### Search results and study selection

Our search detected 213 citations. These included editorials, reviews, letters, or articles regarding other aspects of the radial approach. There were 62 observational studies investigating the feasibility and safety of the radial approach in a series of patients. Moreover, we found 13 studies comparing the radial- and femoral approaches in a cohort of patients that included both elective- and acute cases without reporting separate outcomes regarding the different settings. Twelve studies were included in the final analysis. (Figure 18) These comprised 5 randomized trials involving 516 patients: 266 of the transradial- and 250 of the transfemoral approaches. (76; 91-94) Seven further reports using the registry approach of single- or dual center experiences of primary TRPCI were identified. These included 2808 cases, made up of 1212 transradial- and 1596 transfemoral interventions. (82; 95-100) All studies were published in peer-reviewed journals.

### Clinical results

The radial approach reduced risk for major bleeding by 70% compared to TFPCI (0.77% vs 2.61%, OR: 0.30 [95% CI: 0.16, 0.55] P=0.0001; Figure 19). Reductions in the composite of death, myocardial infarction, and stroke were also significant (3.65% vs. 6.55%, OR: 0.56 [95% CI: 0.39, 0.79] P = 0.01; Figure 20). Pooling the 29 events (2.59%) of 1421 TRPCI and 55 (3.18%) of 1800 TFPCI demonstrated a significant mortality reduction in the case of TRPCI. (OR: 0.54 [95% CI: 0.33, 0.86] P=0.01; Figure 21). There were no differences in procedural time and in time to reperfusion between the two access routes. Fluoroscopic times were longer in case of TRPCI; however, there was significant heterogeneity among studies in these parameters. Access site crossover was less frequent in the case of the transfemoral approach while the total hospital charge, assessed in eight studies was lower in the case of the transradial. (Figure 22)

## Discussion

The present meta-analysis found that transradial coronary intervention is highly effective and safe in the setting of acute myocardial infarction. We demonstrated a significant benefit of using radial access for PCI in MI with respect to major bleeding as well as in major adverse events (MACE). These findings were consistent in the setting of primary- and rescue PCI.

Bleeding events have been demonstrated to be associated with an increased risk of MACE including death and recurrent ischemic events in multiple studies. (83, 84, 101) Though the exact relation between bleeding events and higher mortality is unclear, obviating bleeding seems equally important as recurrent ischemic events after PCI. (102) Possible mechanisms of worse outcome after a bleeding event might include bleeding-induced imbalance of the coagulant/anticoagulant mechanisms, (consumption of the anticoagulant proteins, higher platelet turnover), adverse effects induced by transfusion, and premature cessation of antithrombotic/anticoagulant therapy. (73)

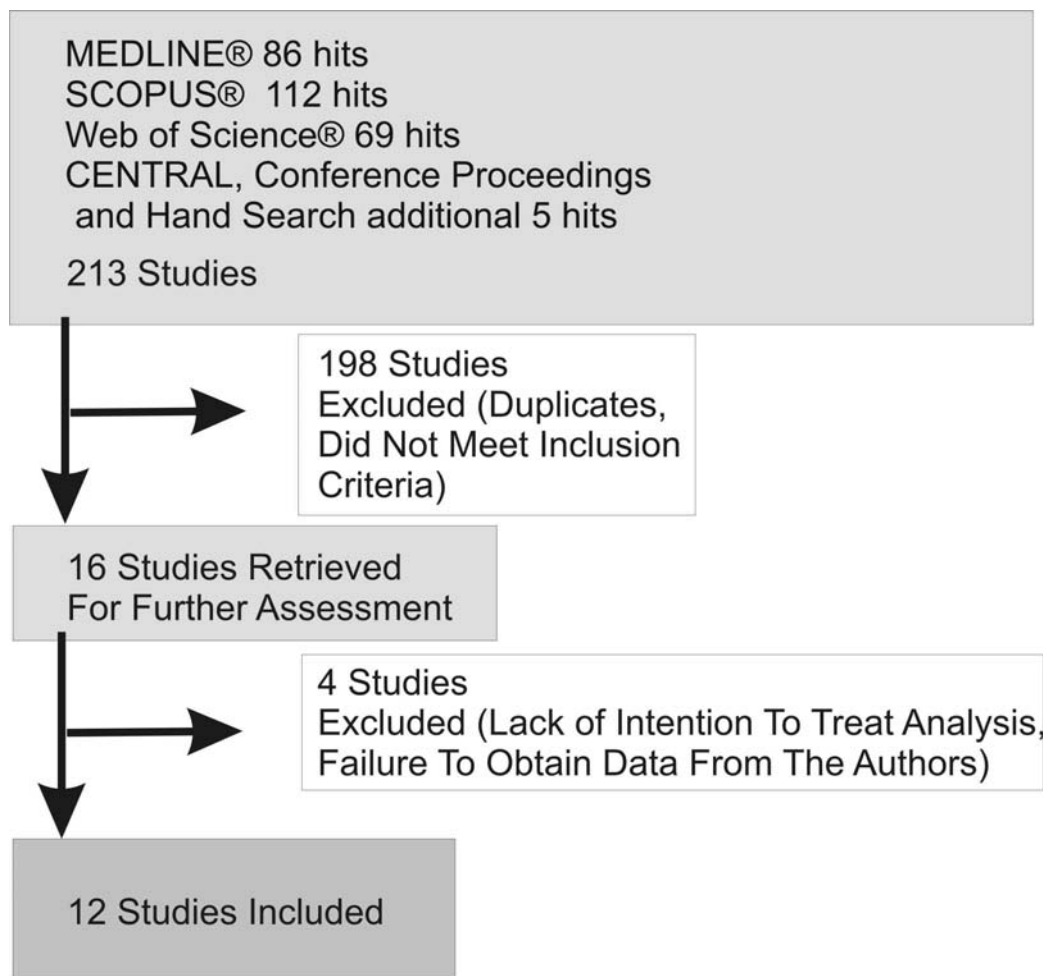


Figure 18. Flowchart of the trials

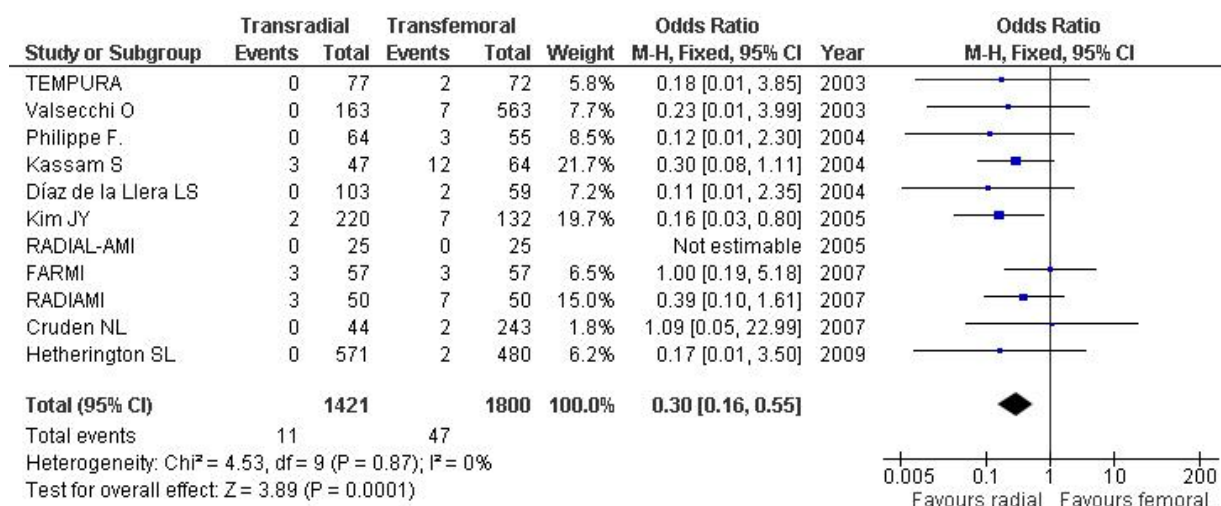


Figure 19. Overall risk of major bleeding. CI: confidence interval; OR: odds ratio

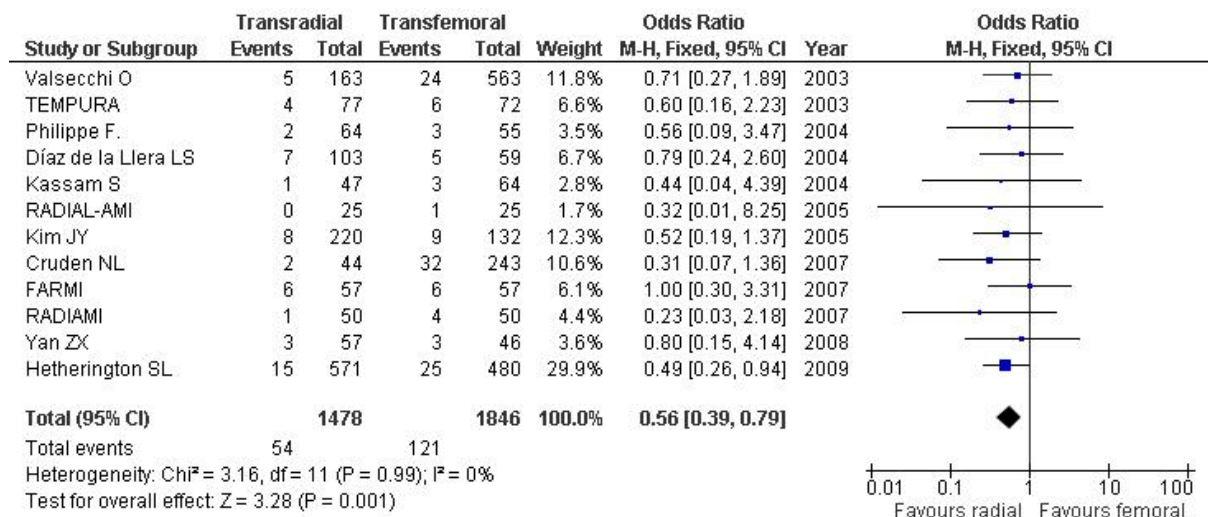


Figure 20. Overall risk of major adverse cardiovascular events (MACE). CI: confidence interval; OR: odds ratio

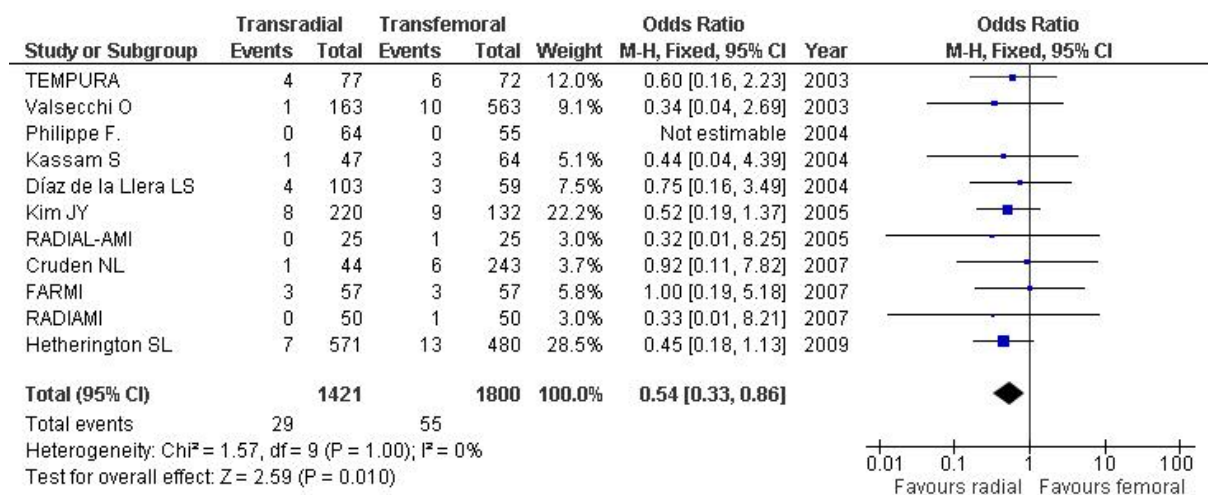


Figure 21. Overall risk of death. CI: confidence interval; OR: odds ratio

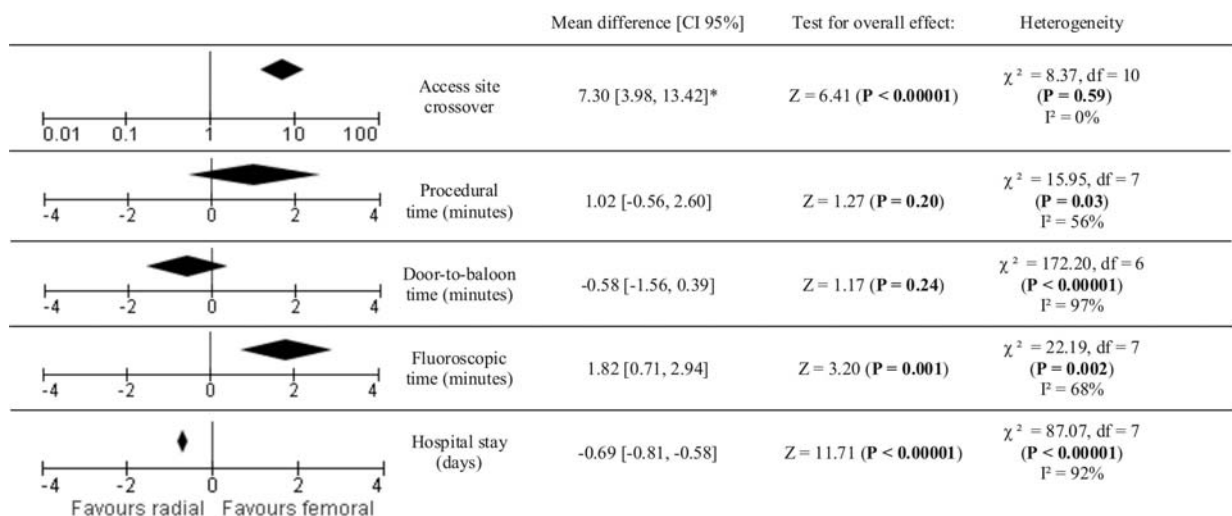


Figure 22. Summary of outcomes of secondary endpoints radial versus femoral access for coronary intervention in myocardial infarction. CI: confidence interval, \* Odds ratio [CI 95%].

As a consequence of the combined- and more potent anticoagulant and antiplatelet medications, bleeding is getting to be more frequent after acute intervention. Arterial puncture used for intervention is a predominant predilection site as the majority of bleeding originates from here. Reduction of the frequency of bleeding and mortality using the transradial approach has been recently demonstrated in a large registry study that included all-comers for PCI. (83) Similar reduction has been demonstrated in transradially treated cases in an observational study that included over a thousand non-ST segment elevation acute coronary syndrome patients. (103) However, distrust supported by technical difficulties, higher failure rate, and increased radiation exposure still limits general acceptance of TRPCI. This uncertainty is grater in the setting of acute MI, when the delay caused by unsuccessful arterial access may adversely affect the clinical outcome. Agostoni et al. reported reduction of the entry-site complications at the expense of more frequent entry failure requiring crossover to a second entry site with significant heterogeneity among studies. (72) They noted that this may have been due to an initial learning curve by cardiologists employing the radial technique that was followed by a progressive equalization in technical skills for both the radial- and femoral approaches through the years. Additionally, not only the initial learning curve but also the constant practice might be important in keeping the complication rate low.

In our analysis, procedural times showed significant variation among studies and TRPCI was not associated either with significantly longer procedural durations or with longer reperfusion times. This underlines the role of expertise and the notion that use of the usual access site in case of emergency might result in shorter procedural length. Altogether the heterogeneity in the different time parameters supports that other factors – most probably experience of the team and skills of the operator as well as the different local protocols – affect them more than the choice of the arterial puncture.



## Conclusion

The current meta-analysis of the literature demonstrated that transradial coronary intervention improves clinical outcomes by reducing major bleeding and postprocedural ischemic complications in patients with acute myocardial infarction. Thereby, this access site might maximize the benefits and reduce the potential harm of the aggressive antiplatelet and anticoagulant therapy required among these patients.

## 6. Novel findings

Based on the results of the cited experiments and studies, our major novel findings can be summarized as follows:

- Results of our measurements with light transmission aggregometry confirmed that the response to a fixed-dose aspirin and thienopyridines are not uniform between patients. While the response to aspirin follows a bimodal, yes-or-no distribution, the efficacy of thienopyridine therapy is normally distributed. (Figure 12). Likewise, it is rationale to use the term “aspirin resistance” based on laboratory assessments; however, the term “high-post treatment platelet reactivity” (HPPR) is more appropriate compared to “clopidogrel resistance” in case of thienopyridines as the measured effect (P2Y12 receptor inhibition and ADP-reactivity) seems dose-dependent.
- The significant correlation with VASP validates LTA for monitoring the efficacy of P2Y12 receptor inhibition (Figure 9); however, there might be clinically meaningful differences in the results in certain individuals. Indeed, 6-minute late aggregation is not superior to other estimates of LTA in monitoring the efficacy of P2Y12-receptor inhibition.
- Low response to clopidogrel and high post-clopidogrel platelet reactivity is associated with a higher risk of recurrent ischemic events in low-risk patients after percutaneous coronary interventions (Figure 13). Patients above the median value of maximal aggregation (>33%) had a 6.8-fold HR to MACE.
- Doubling the maintenance dose of clopidogrel to 150 mg intensifies the efficacy of clopidogrel therapy in patients with high platelet reactivity (Figure 16). However, less than 50% of the patients with HPPR might return to normal platelet reactivity (Figure

15B). Low platelet disaggregation and acute coronary syndrome are independent predictors of poor response to 150 mg clopidogrel (Table 7).

- In patients with AMI receiving dual or triple antiplatelet therapy, using radial artery as an access site to perform primary PCI not only reduces access site complications and major bleeding but also major adverse cardiac events and mortality.

## 7. Conclusions and perspective

We started our examinations in an era when only limited evidence were available on the rationale and consequences of monitoring the efficacy of antiplatelet therapy in patients after percutaneous coronary intervention. At that time, “aspirin resistance” was a popular and well-known term among clinicians; however, due to heterogenic definitions and non-standardized laboratory assessments, the range of aspirin resistance varied largely between authors. (43) One should be aware that the wide range of the prevalence of aspirin resistance cannot be explained by the different risk profile of the patient groups. It should be attributed to the arbitrary-used definitions and non-specific laboratory tests that might have lead to both over- and underestimation of the incidence of aspirin resistance in many examinations.

As landmark studies demonstrated a significant clinical benefit of adding thienopyridines to aspirin monotherapy after coronary stent implantation, dual antiplatelet therapy became gold-standard in patients after PCI. (5-8) Knowing that clopidogrel has less haematological side-effects than ticlopidine, the former has become the thienopyridine of choice.

As thrombotic events still occurred in spite of the dual antiplatelet therapy, extensive research started to explore the individual response to clopidogrel. Early studies demonstrated large inter-individual differences in response to a fix-dose clopidogrel, and similar to aspirin, the term “clopidogrel resistance” was created and widely applied to refer for patients with inappropriate response. (45-47) However, compared to aspirin, there are major differences in testing the response to clopidogrel therapy. Clopidogrel is a platelet surface receptor blocker while aspirin is an intracellular enzyme inhibitor. Using a specific agonist (ADP) for testing aggregation is reliable in reflecting the inhibition of the P2Y<sub>12</sub> receptor. Though our results showed that ADP-stimulated LTA is not fully P2Y<sub>12</sub>-specific as it was also influenced by P2Y<sub>1</sub>-receptor activation, all aggregation values were in significant correlation with VASP-PRI. In contrast, the recently available assays to measure the efficacy of aspirin (urinary

thromboxane and serum thromboxane levels, PFA-100 collagene-epinephrine closure time, LTA with arachidonic acid, adrenalin or collagen stimuli) are varying largely in determining the prevalence of aspirin resistance and correlating poorly amongst themselves making it difficult compare and impossible to interpret their results. (40) These facts emphasise that not all methods are equal in estimating antiplatelet response and there are substantial differences in reliability between assays in monitoring the efficacy of aspirin and clopidogrel treatment. (44) Based on our results, the CARAT TX4 aggregometer with ADP 5  $\mu$ M stimuli shows fair correlation to VASP measurements in monitoring the efficacy of clopidogrel therapy. Without doubting its shortcomings, LTA-assessment is a validated and valuable tool for testing clopidogrel's response.

By measuring the efficacy of antiplatelet therapy in patients after PCI, we found that the distribution of clopidogrel response follows a normal, Gaussian distribution pattern. This is in line with the findings of several other studies and it is not considered as a laboratory artefact. (42, 45) This distribution pattern is quite frequent in processes that are under both environmental and polygenetic control. Clopidogrel is a prodrug that needs to be metabolized into a thiol derivate to inhibit P2Y<sub>12</sub> ADP receptor. (20) However, more than two thirds of the absorbed clopidogrel is converted into a pharmacologically inactive carboxyl metabolite by blood esterases, whereas only a small proportion might be metabolized into an active derivate through the cytochrome enzyme system (CYP2C19, 2C9, 3A4, 3A5, 1A2, 2B6). Importantly, the loss-of-function alleles (\*2, \*3, \*4) of the CYP2C19 enzyme, that plays a pivotal role in the bioactivation process is very frequent in the Caucasian population (22) Moreover, the absorption of clopidogrel might also differ between patients according to the genetic polymorphisms of the P-glycoprotein. (104) Clinical factors like diabetes, acute coronary syndrome and renal insufficiency might also impair the efficacy of clopidogrel therapy. (61) As a result, high post-clopidogrel platelet reactivity (HPPR) persists in a

substantial proportion of patients despite receiving the recommended dose of clopidogrel. As the distribution of platelet aggregation values and VASP-PRI is normal, the term “elevated or high platelet reactivity” is more appropriate than “clopidogrel resistance” as the effect seems dose dependent. (105)

Growing body of evidence shows that high platelet reactivity is a risk marker for recurrent thrombo-ischemic events after PCI. (48-55) In our study recruiting 134 patients, ADP-stimulated  $\text{Agg}_{\text{max}}$  values were significantly associated with clinical outcome. Dividing patients into quartiles according their  $\text{Agg}_{\text{max}}$  values showed that those in the two higher quartiles had a 6.8-fold risk to MACE compared to those in the two lower ones. (59) The worse prognosis of patients in the two higher quartiles of residual platelet aggregation values was independently demonstrated by the EXCELSIOR study in 802 patients. They registered a 6.7-fold higher risk for death, MI or urgent TLR among patients above the median value of platelet aggregation. (50) A meta-analysis also confirmed the prognostic significance of high platelet reactivity in patients after PCI. (54) Despite these results, it is unclear whether high platelet reactivity is a marker for worse prognosis (risk marker) or a modifiable parameter (risk factor). It would be of enormous importance to know whether patients with HPPR might return to normal platelet reactivity with alternative antiplatelet strategies. Results of a recent work of Bonello et al. confirmed that in patients with high platelet reactivity, repeated loading doses of 600 mg clopidogrel were able to override low response to clopidogrel in the majority of patients. (58) However, findings of other studies suggest that returning to the standard dose of clopidogrel in these patients resumes high platelet reactivity. (68) In case of the maintenance-phase treatment, there are a few studies that have demonstrated the pharmacological benefit of doubling the maintenance dose of clopidogrel. (64-69) Our results supported that giving 150 mg clopidogrel for patients with HPPR enhanced platelet inhibition. Notably, the degree of platelet reactivity after 150 mg clopidogrel did not reach that of good

responders and less than 40% of the patients returned to normal platelet reactivity with the elevated maintenance dose. Analysing possible determinants of response to 150 mg clopidogrel revealed platelet disaggregation and acute coronary syndrome as independent predictors of poor response. Acute coronary syndrome was associated with a 1.7-fold, while low disaggregation with a 6.1-fold absolute risk for persisting with HPPR after 150 mg clopidogrel. Low disaggregation had a 92% negative predicting value emphasising that these patients are not likely to benefit from dose shift. These results might carry important clinical implications as clinicians might be able to choose optimal candidates for a 150-mg maintenance dose based on the clinical status and the result of a LTA assessment.

Based on these findings, the potency of the platelet inhibition can be influenced by the administered dose of clopidogrel. However, many of the patients with HPPR would require such high loading and maintenance doses to achieve optimal platelet reactivity that are not tolerable and also not financed by our health care system. (70) These individuals might be the candidates for alternative, more potent platelet inhibitors. One of the most important alternatives of clopidogrel is prasugrel that is recently under approval in Hungary. Based on the results of the TRITON-TIMI38 trial, the more potent antiplatelet therapy in ACS was associated with significantly less myocardial infarctions and stent thromboses. (32) Ticagrelor, a reversible and also potent, direct-acting ADP-receptor antagonist has also demonstrated a 16% reduction in the composite of MI, cardiovascular death and stroke in the PLATO trial. (33)

Notably, the administration of prasugrel increased bleeding complications including spontaneous, fatal bleeds highlighting that the uniformly intensified antiplatelet therapy might do harm to some patients. As bleeding is an equally important predictor of morbidity and mortality as ischemia, preventing bleeding events is of essential importance. (82, 108) Patients admitted to primary PCI due to STEMI represent a high-risk group of both bleeding

and ischemia. The double or triple antiplatelet therapies with excessively dosed anticoagulants are important causes of bleeding events among these patients. However, choosing the radial artery as the preferred access site dramatically lowers not only periprocedural bleeding complications but also major adverse cardiac events and mortality. (106) Many operators are fearing to puncture the radial artery for PCI in the setting of AMI, believing that it prolongs the intervention and increases the amount of contrast and radiation exposure. Our meta-analysis confirmed that the major cause of the longer procedural times and the higher radiation exposures is the learning-curve required to master the technique. This aspect was indicated by the significant heterogeneity between the included studies. Based on these results, the radial approach is also a way to maximize the benefits of an intensified antiplatelet therapy in the peri-interventional period of primary PCI.

Concluding these findings, there is a great need for an individualized antiplatelet therapy for patients in order to maximize the anti-ischemic benefits and minimize bleeding risk. Beyond clinical presentation and the genetic constellation, platelet function testing might help to find a therapeutic window for each antiplatelet agent. The novel, more potent antiplatelet agents will be essential tools to customize antiplatelet therapy to the patient's needs as suboptimal response to clopidogrel is common. The clinical relevance of an individualized antiplatelet regimen needs to be tested and supported in further clinical trials.



## 8. References

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## 9. Publication list

### I. Topic-related international articles

1. **Aradi D**, Konyi A, Palinkas L, Berki T, Pinter T, Tahin T, Horvath I, Papp L, Komocsi A. Thienopyridine therapy influences late outcome after coronary stent implantation. *Angiology* 2008; 59:172-8.  
IF: 1.122
2. **Aradi D**, Vorobcsuk A, Lenkey Zs, Horváth IG, Komócsi A. Low platelet disaggregation predicts poor response to 150 mg clopidogrel in patients with elevated platelet reactivity. *Platelets* 2009; accepted for publication. ID: CPLA-2009-0136.  
IF(2008): 2.271
3. Vorobcsuk A, Kónyi A, **Aradi D**, Horváth IG, Ungi I, Louvard Y, Komócsi A. Transradial Versus Transfemoral Percutaneous Coronary Intervention in Acute Myocardial Infarction. Systematic Overview and Meta-Analysis. *Am Heart J* 2009. accepted for publication.  
IF(2008): 4.285
4. **D. Aradi**, Pinter T, Horvath IG, Komocsi A. Variability in response to clopidogrel: where is the threshold for 'low response'? *Eur Heart J* 2007; 28:515-6. *Letter*  
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5. **D. Aradi**, T. Paska, M. Tóké-Füzesi, A. Komócsi. Monitoring the Efficacy of Antiplatelet Therapy: All Methods are Equal, but Some Methods are More Equal than Others? *Am Heart J* 2008; 155:e33. *Letter*  
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6. **D Aradi**, A Vorobcsuk, A Komócsi. "Optimizing clopidogrel therapy before stent implantation: should clinical setting be taken into account?" *J Am Coll Card* 2008; 52:1349. *Letter*  
IF: 11.438

## II. Non-topic related international articles

1. Lenard L, **Aradi D**, Donauer E. Migrating Kirschner wire in the heart mimics acute coronary syndrome. *Eur Heart J* 2009; 30:754.  
IF(2008): 8.917
2. Komocsi A, Simor T, Toth L, Szabados S, Magel F, Pinter T, **Aradi D**, Konyi A, Horvath IG, Papp L. Magnetic resonance studies in management of adult cases with Bland-White-Garland syndrome. *Int J Cardiol.* 2007; 123:e8-11.  
IF: 2.234

Cumulative Impact Factor: 41.84 (without letters: 18.83)



## III. International abstracts

1. **Aradi D**, Konyi A, Palinkas L, Berki T, Pinter T, Tahin T, Horvath I, Papp L, Komocsi A. Efficacy of thienopyridine therapy influences late outcome after coronary stent implantation. (TCT2006)  
*Am J Cardiol* 2006; **98**:(8,Suppl.1)S200-S207 Abstract No. 517.  
IF: 3.015
2. **D. Aradi**, T. Pinter, A. Konyi, T. Tahin, I. Horvath, L. Papp, A. Komocsi. Efficacy of thienopyridine therapy influences late outcome after coronary stent implantation. (ESC 2006)  
*Eur H J* 2006;27(Suppl):750. Abstract NoP4558.  
IF: 7.286
3. **D. Aradi**, T. Pinter, I. Horvath, A. Konyi, A. Komocsi. Low response to thienopyridine therapy after stent implantation is associated with the need for target vessel revascularization in elective but not in acute coronary syndrome patients. (ESC2007) *Eur Heart J Suppl.* 2007;28: 497-723. Abstract No. P4005.  
IF: 7.286
4. **D. Aradi**, Iván G. Horváth, Tünde Pintér, Attila Kónyi, András Komócsi. Low Platelet Disaggregation Predicts Impaired Response to High Maintenance Dose of Clopidogrel in Patients with High Post-Clopidogrel Platelet Reactivity. (TCT2008)  
*Am J Cardiol* 2008; 102(8): Suppl. Abstract No.: P45.
5. **D. Aradi**, A. Vorobcsuk, M. Tokes-Fuzesi, T. Magyarlaki, IG. Horvath, A. Komocsi. Monitoring P2Y12 receptor inhibition with light transmission aggregometry: a comparison with vasodilator stimulated phosphoprotein phosphorylation assay. (ESC2009). *Eur Heart J Suppl.* 2009. Abstract No: 87661.

## IV. Hungarian abstracts

1. **Aradi D.**, Pintér T., Horváth I., Berki T., Pálincás L., Komócsi A. A thrombocytáaggregáció és adhéziós integrinek kapcsolata az eseménymentes túléléssel stentimplantáció után. *Cardiologia Hungarica* 2006; 36: A42.
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3. **Aradi D.**, Pintér T., Horváth I., Kovács E., Magyarai B., Kónyi A., Komócsi A. The impact of diabetes mellitus on the efficacy of combined antiplatelet therapy after coronary stent implantation. *Cardiologia Hungarica* 2007.
4. **Aradi D.**, Pintér T., Kónyi A., Horváth I., Kovács E., Magyarai B., Komócsi A. 150 mg fenntartó dóziszú clopidogrel csökkenti a magas reziduális thrombocytá-reaktivitás gyakoriságát percutan coronaria intervenció után. *Cardiologia Hugarica* 2008; 38: B36.
5. Magyarai Balázs, Horváth Iván, Kónyi Attila, Komócsi András, Pintér Tünde, Kovács Erik, **Aradi Dániel**, Papp Lajos: A gyógyszerkibocsátó stent felhasználás változása a 2006-os ESC tükrében. *Cardiologia Hugarica* 2008; 38: B61
6. Pintér Tünde, **Aradi Dániel**, Komócsi András, Kovács Erik, Kónyi Attila, Magyarai Balázs, Horváth Iván, Francesco Burzotta, Darius Dudek: PIHRATE (Polish-Italian-Hungarian randomized thrombectomy trial) tanulmány eredményei. *Cardiologia Hugarica* 2008; 38: B86.
7. Kovács Erik, Komócsi András, Kónyi Attila, Pintér Tünde, **Aradi Dániel**, Magyarai Balázs, Horváth Iván: Az On-site záróeszköz betanulási fázisának vizsgálata az artéria femorális behatolásból végzett diagnosztikus koronarográfián átesett betegek körében. *Cardiologia Hugarica* 2008; 38: B59

8. **Aradi D**, Vorobcsuk A, Sayour A, Magyarlaki T, Tókécs-Füzesi M, Rideg O, Horváth IG, Komócsi A.: Optikai aggregometria mérési eredményeinek összevetése specifikus P2Y<sub>12</sub> receptor gátlás áramlási citometriás meghatározásával. *Cardiologia Hungarica* 2009; 39: A19
  
9. Komócsi A, Vorobcsuk A, Ungi I, **Aradi D**, Horváth IG, Kónyi A.: Transzradiális PCI akut miokardiális infarktus ellátása során – szisztematikus irodalmi áttekintés és metaanalízis. *Cardiologia Hungarica* 2009; 39: A1.

## V. International Poster Presentations:

1. **Aradi D**, Konyi A, Palinkas L, Berki T, Pinter T, Tahin T, Horvath I, Papp L, Komocsi A. Efficacy of thienopyridine therapy influences late outcome after coronary stent implantation. Transcatheter Cardiovascular Therapeutics 2006 (TCT'06), Washington DC.
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