Genetic approach to the laboratory efficacy of anti-platelet treatment in patients after coronary stent implantation

Ph.D. thesis

by

Orsolya Rideg M.S

Supervisors

Attila Miseta M.D., D.Sc., Ph.D.

and

András Komócsi M.D., Ph.D.

Head of the Ph.D. program

Attila Miseta M.D., D.Sc., Ph.D.

University of Pécs, Faculty of medicine

Pécs, 2011

Contents

1. Abbreviations 3
2. Introduction 6
    2.1 Stent thrombosis 7
    2.2 Prevention of instent restenosis 8
    2.3 Adjuvant therapy of coronary stent implantation 9
    2.4 Interindividual differences in response to anti-platelet therapy 14
    2.5 Genetic variations of ABCB1, Cytochrome P450 2C19 (CYP2C19), and Paraoxonase 1 (PON1) genes 16
3. Aims 20
4. Methods 22
    4.1 Laboratory methods to assess anti-platelet efficacy 22
    4.2 Genetic analysis of the functionally relevant ABCB1, CYPC19 and PON1 allelic variants 28
    4.3 Real Time PCR (RT PCR) 29
    4.4 Study design and patient population 32
    4.5 Definition of high on-clopidogrel platelet reactivity (HPR) in our clinical studies 34
    4.6 Statistical Analysis 34
5. Results 37
    5.1 Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: Systematic review and meta-analysis 37
    5.2 Comparison of conventional aggregometry (LTA) with vasodilator stimulated phosphoprotein phosphorylation assay (VASP) 47
    5.3 Determining the impact of genetic variants on post-clopidogrel platelet reactivity in patients after elective percutaneous coronary intervention 51
6. Discussion 62
7. Novel findings of the thesis 67
8. Limitations 68
9. Summary 70
10. List of Publications 72
    10.1 Topic related 72
    10.1.1 International articles 72
    10.1.2 Hungarian abstracts and posters 73
    10.2 Non Topic related 75
11. Acknowledgements 79
12. References 80

1. **Abbreviations**

AC	Adenil cyclase
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
Agg	Aggregation
Agg_{max}	Maximum aggregation
Agg_{late}	Late aggregation
APT	Anti-platelet therapy
ARC	Academic Research Consortium
ASA	Acetylsalicylic acid
AUC	Area under curve
CABG	Coronary-artery bypass grafting
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CIE	Composite ischemic event
COX	Cyclooxygenase
CYP450	Cytochrome P450
CV	Cardiovascular
DAG	Diacylglycerol
DAPT	Dual anti-platelet therapy
DES	Drug eluting stent
disAgg	Disaggregation
GOF	Gain-of-function
GPI	Glycoprotein receptor inhibitor

HPR	High on-platelet reactivity
HR	Hazard ratio
INS	Insulin-treated diabetes mellitus
IP3	Inositol triphosphate
ISR	In-stent restenosis
LC RT PCR	LightCycler Real Time PCR
LOF	Loss-of-function
LTA	Light Transmission Aggregometry
MACE	Major adverse cardiovascular events
Mdr1	Multidrug resistance 1
MFI	Mean fluorescence intensities
MFI_c	Corrected Mean fluorescence intensities
MI	Myocardial infarction
MLCK-P	Myosin light chain kinase
OAD	Oral antidiabetic-treated diabetes mellitus
OR	Odd ratio
PAD	Peripheral artery disease
PCI	Percutaneous coronary interventions
PGE_1	Prostaglandin E1
Pgp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
POBA	Percutaneous ballon angioplasty

PON1	Paraoxonase-1 enzyme
PPP	Platelet poor plasma
PRI	Platelet reactivity index
PRP	Platelet rich plasma
ROC	Receiver-operator characteristic curve
SNP	Small nucleotide polymorphism
ST	Stent thrombosis
Tm	Melting temperature
TTP	Thrombotic thrombocytopenic purpura
TVR	Target vessel revascularization
TXA_2	Thromboxane A_2
UFH	Unfractionated heparin
VASP-PRI	Vasodilator-stimulated phosphoprotein phosphorilation assay
WB	Whole blood

2. Introduction

Coronary artery disease (CAD) is known to be a very heterogeneous disease of multi-factorial origin affecting millions world-wide and being the top leading cause of death among the middle-aged population. Due to the actuality and complexity of the subject the area is being widely studied. In recent decades new medications, treatment protocols and follow up methods were developed for its management. Coronary-artery bypass grafting (CABG), introduced in 1968, used to be the only method of coronary revascularization until 1977 when Andreas Grüntzig performed percutaneous ballon angioplasty (POBA). Since then both technologies underwent evolution exploiting numerous advantages of minimal invasive intervention. Substantial technological and pharmacological advances increased the feasibility and procedural success. In parallel with the technological improvement the adjunctive pharmacology had substantial improvement involving development of new drugs with novel targeted mechanisms, refined indications and risk stratification-based treatment algorithms.

In the 1990s the development of coronary stents led to reduced rates of complications of balloon angioplasty (POBA), and percutaneous coronary interventions (PCI) have revolutionized the treatment of ischaemic heart disease. However some short and longer term complication has been realized. (1;2) Using anti-platelet therapy in patients after PCI radically reduced the rate of ischaemic events. It is of note that promising results are associated in some clinical situations with a need for higher degree inhibition of platelet activation using more potent anti-platelets, as well as intensified anti-platelet and anticoagulation protocols. Despite the efforts, acute stent thrombosis (ST) and instent restenosis (ISR), both resulting in target-vessel failure after PCI, still occur. It underscores that optimizing anti-platelet therapy is still not achieved in a proportion of patients and emphasizes the complexity of the question. Adjusting the sensitive balance of the anti-platelet therapy, to being well effective, but as well

avoiding the risk of serious side effects, like peri-procedural bleeding complications, is of great importance. Considering that individuals may have different needs for anti-platelet action as well as different risk for bleeding, points toward the need for an individualized therapeutic regime. Significant interindividual differences in response to anti-platelet therapy have recently been recognized with supposed environmental, clinical, pharmacokinetic and genetic background. While lack of standardization of platelet function assays and well defined cut off values represent further difficulties of the subject. (1-5)

2.1 Stent thrombosis

Stent thrombosis (ST) is a dreadful complication after coronary stent implantation. (6) ST means blood clot formation in the previously implanted stent and as a consequence it encounters myocardial infarction associated with high mortality. By reason of terminological heterogeneity, in 2006 the Academic Research Consortium (ARC) proposed consensus categories for ST, based on event probability and level of evidence, also known as Glasgow-classification. Alternatively ST could be also classified based on the timing of occurence after PCI (early: acute, sub-acute; late; very late ST). ST is a multi-factorial event; in that both procedural (under-expansion, malposition 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 anti-platelet therapy, and possibly inefficacious APT) play substantial role. (7) The risk of acute ST is greatest in the early phase with approximately 2% in the initial month for all types of stents, while overall incidence of ST is around 2-4%. (8;9)

The use of dual anti-platelet therapy (DAPT) in patients after percutaneous coronary interventions (PCI) is supported by strong body of evidence, demonstrating reduction of the risk of ischaemic complications and better overall outcome. Inhibiting platelet function is a key therapeutic principle to reduce major adverse cardiovascular events (MACE), but several

trials and clinical registries have also shown that this advantage may be offset in certain circumstances like by an increased risk of bleeding complications after cardiovascular surgery. (10) As a consequence, the individual ischaemic and bleeding risk of a patient must be taken into consideration to select the appropriate platelet inhibitory regimen offering the best benefit-risk ratio. (11)

2.2 Prevention of instent restenosis

Instent restenosis (ISR) is a chronic process that occurs when the treated vessel becomes blocked again after PCI. Similar to ST, ISR also a multi-factorial disorder in which both procedural (under-expansion, malposition of stents, long stented segment), clinical (acute coronary syndrome, impaired renal function, diabetes) play substantial role. (12;13) It is typically seen 3 to 6 months after the initial procedure. When a stent is placed in a blood vessel new tissue grows inside the stent, covering the struts. Initially this is a favorable effect by developing a normal lining over stent allowing smooth blood flow over the stented area without provoking clot formation. (14;15) In time, scar tissue may arise underneath the new healthy lining obstructing the blood flow by producing an important blockage. With the implantation of bare metal or cobalt-chromium stents, ISR occurred up to 20 to 40% of patients after PCI. In the past years, drug-eluting stents (DES) proved to be extremely successful in preventing in-stent restenosis. (8;9) The anti-inflammatory, anti-proliferative and/or cytostatic drugs that are released from stents to the vessel wall decreased the incidence of angiographic in-stent restenosis below 10%. On the other hand the antiproliferative effect also delays the healing process and thus the coverage of the sruts. This results in a longer period of risk for stent thrombosis and longer need for preventive anti-platelet therapy.

2.3 Adjuvant therapy of coronary stent implantation

Anti-platelet agents

Platelets are relevant in the process of atherothrombosis and arterosclerosis. (14;16) The surface of the resting platelet carries a great variety of specialized and highly regulated transmembrane receptors for different agonists and ligands. Lesion of the endothelium through the rupture of atherosclerotic plaques, or during PCI by damaging the intimal layer integrity, permits platelets to contact subendothelial factors that promote thrombocyte adhesion and aggregation cascade. Thus inhibition of platelet activation and aggregation is one of the major pharmacological goals in our therapeutic regimen. Currently, three main groups of drugs are used after and before coronary stent implantation. These targeted substances inhibit different steps in platelet activation and/or aggregation.

2.3.1 GP-IIb/IIIa receptor antagonists

GP-IIb/IIIa inhibitors (GPI) are intravenously administered anti-platelet agents. They prevent platelet aggregation and thrombus formation by suppressing GP-IIb/IIIa receptor on the activated platelet surface. They are frequently used in high-risk ACS patients who do not have active bleeding and it is also recommended in patients with acute myocardial infarction admitted for primary PCI. (8;17;18) However, they also increase the rate of bleeding events. (19) The available formulations are synthetic, small-sized particles (tirofiban and eptifibatide) and monoclonal antibodies (abciximab). Contrary to intravenous molecules that are supported by several studies and guidelines, the development of IIb/IIIa targeted oral APT was hampered by paradoxical higher thrombosis risk, increased mortality and ischemic events in early trials. (20)

2.3.2 Cox-1 inhibition: Aspirin

Cyclooxigenase (COX) enzyme catalyzes the conversion of arachidonic acid to series of prostanoids, most importantly to the highly bioactive, aggregation inductor and vasoconstrictor thromboxan A_2 (TXA_2). Aspirin's active substance, the acetylsalicylic acid (ASA), irreversibly inactivates megakaryocytes' and platelets' cyclooxygenase (COX) activity by selectively acetylating the hydroxyl group of a single serine residue of the enzyme. Currently three types of COX isoenymes are known (COX-1, COX-2 and COX-3) of which platelets only express COX-1. According to the results of a large meta-analysis (287 studies involving 135000 patients) aspirin therapy reduced the combined outcome of any serious vascular event by about one quarter; non-fatal myocardial infarction was reduced by one third, non-fatal stroke by one quarter, and vascular mortality by one sixth. (22) Likewise aspirin therapy is often recommended lifelong especially after PCI. Permanent inactivation of platelets' COX enzyme by low to medium dose aspirin (32.5–75 mg daily) is well tolerated in the majority of patients, but it must be considered that aspirin also amplifies the risk of bleeding (mainly gastrointestinal bleeding and haemorrhagic stroke). (21;22) Thus, the possible value of aspirin in reducing thrombosis may be weighed against the risk of bleeding, with the greatest value being in those at highest cardiovascular risk.

2.3.3 ADP-receptor antagonists

Since the ADP receptor, $P2Y_{12}$ transmitted effect plays a pivotal role in the amplification of platelet aggregation leading to a stable occlusive thrombus, inhibition of the receptor was an early focus in anti-platelet drug development. Ticlopidine, clopidogrel and prasugrel are structurally related thienopyridines with platelet inhibitory properties. (23) These inactive pro-drugs require metabolic activation of the hepatic cytochrome P450 (CYP450) pathway to become active metabolites. Active derivatives of thienopyridines irreversibly and covalently

bind to the $P2Y_{12}$ receptor on platelets membrane and block the receptor-induced reduction in intracellular cAMP, resulting in reduced aggregation of the platelets. (24) Permanent modification of an ADP receptor by thienopyridines is consistent with time-dependent, cumulative inhibition of ADP-induced platelet aggregation on repeated daily dosing and with slow recovery of platelet function on drug withdrawal.

2.3.3.1 Ticlopidine

The first widely used thienopyridine was ticlopidine. (25) Due to its inconvenient pharmacological profile (b.i.d, saturation after 5 days), and side effect profile it has been substituted by its second generation analog; clopidogrel. Despite the early promising results, showing ticlopidine's potential to reduce cardio vascular events, due to the more serious side effects compared to clopidogrel, as bone-marrow toxicity and fatal thrombotic thrombocytopenic purpura (TTP), the use of ticlopidine in the last years has been diminished. Ticlopidine currently used in patients in whom aspirin is not tolerated or in whom dual anti-platelet therapy is not recommended.

2.3.3.2 Clopidogrel

Clopidogrel is a pro-drug that is rapidly absorbed after oral administration. Approximately 85% of the absorbed drug is converted into a pharmacologically inactive carboxyl metabolite by blood esterases. (26) Whereas only the remaining 15% is metabolized into an active derivative through the cytochrome enzyme system (CYP2C19, 2C9, 3A4, 3A5, 1A2 and 2B6) and paraoxonase-1 (PON1) enzyme. (27;28) During the last decade clopidogrel was tested in numerous large clinical trials for safety and efficacy of the drug as well as the relevancy of dual anti-platelet therapy (DAPT) in combination with aspirin. (29;30) As a consequence, clopidogrel therapy is recommended in a wide spectrum of patients with ischemic heart disease. By means of the CAPRIE trial, that compared clopidogrel with aspirin in reducing

risk of vascular events in patients with atherosclerosis, clopidogrel has been proven to be superior to aspirin. (29) In the CURE study clinical benefits of aspirin-clopidogrel DAPT, in patients with unstable angina, non-segment elevation ACS, were emphasized. It showed a reduction in composite endpoint of cardiovascular death (CV), myocardial infarction (MI) or stroke by approximately 20%. (30) As a supplement of the CURE trial PCI-CURE and CREDO trials, on the one hand, demonstrated, that using clopidogrel in addition to pretreatment with aspirin, in PCI-treated patients was associated with a risk reduction ratio of 31% for CV/MI. On the other hand it confirmed the clinical efficacy of prolonged clopidogrel therapy beyond 30 days up to 9-12 months after stent implantation. (31;32) Beside the promising results, an increase in bleeding complications was also registered, comparing dual anti-platelet treatment to aspirin monotherapy. This must take into consideration when tailoring the intensivness of anti-platelet therapy to achieve the maximum efficacy with the lowest harm to the patient. (Figure 1.)

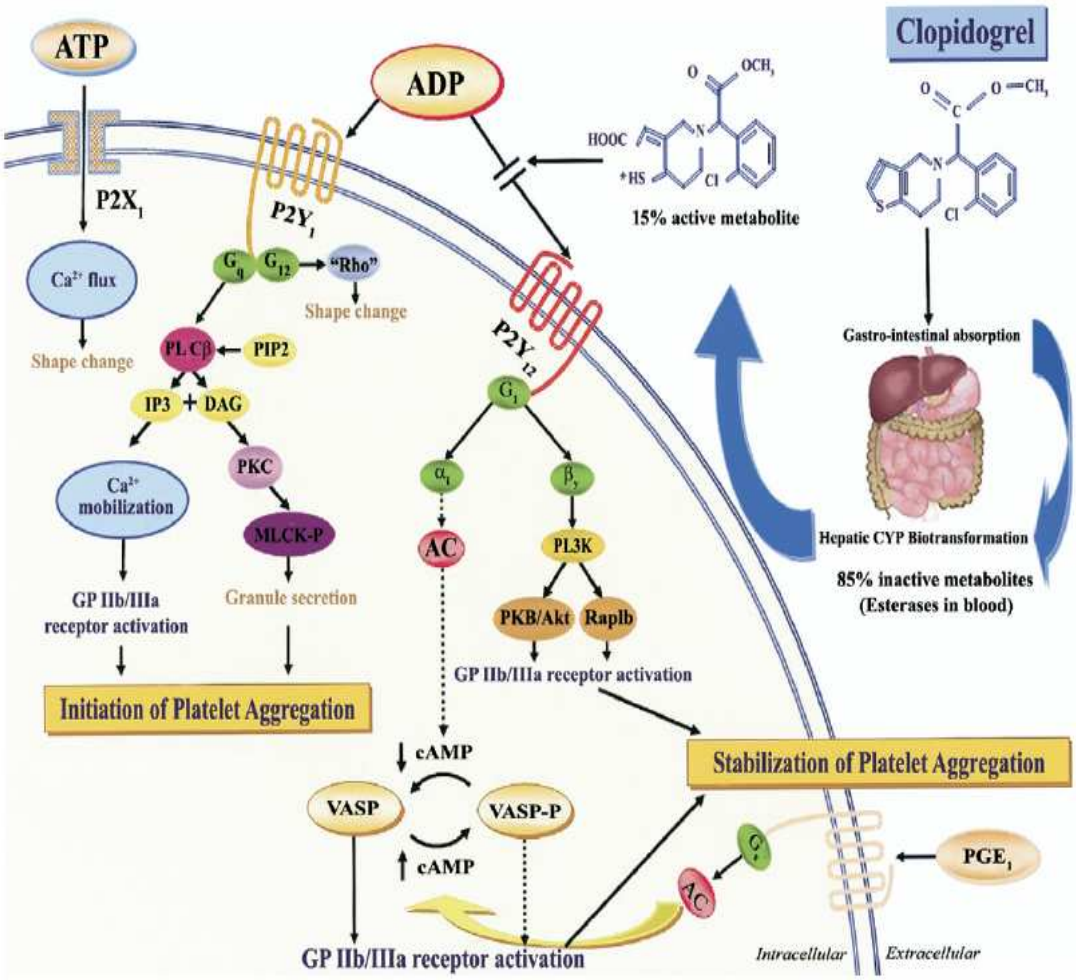


Figure 1. Mechanism of clopidogrel action in the platelet.

(Dominick J Angiolillo et al: Variability in individual responsiveness to clopidogrel;

J Am Coll Cardiol; 2007)

Active metabolite of clopidogrel is generated by the cytochrome P450 (CYP) system in the liver. The active metabolite irreversibly inhibits the adenosine diphosphate (ADP) $P2Y_{12}$ receptor. Activation of the $P2X_1$ and $P2Y_1$ receptors leads to alteration in shape and initiates a weak and transient phase of platelet aggregation. The $P2X_1$ mediates extracellular calcium influx and utilizes adenosine triphosphate (ATP) as an agonist. The binding of ADP to the G_q-coupled $P2Y_1$ receptor leads to activation of phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol triphosphate

(IP3) from phosphatidylinositol bisphosphate (PIP2). Diacylglycerol activates protein kinase C (PKC) leading to phosphorylation of myosin light chain kinase (MLCK-P); IP3 leads to mobilization of intracellular calcium. The $P2Y_1$ receptor is coupled to another G-protein, G_{12}, which activates the "Rho" protein and is believed to lead to change in platelet shape. The binding of ADP to the G_i-coupled $P2Y_{12}$ receptor liberates the G_i protein subunits α_i and ßγ and results in stabilization of platelet aggregation. The α_i subunit leads to inhibition of adenylyl cyclase (AC), which reduces cyclic adenosine monophosphate (cAMP) levels. This, in turn, diminishes cAMP-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (VASP-P). The status of VASP-P modulates glycoprotein (GP) IIb/IIIa receptor activation. The subunit ßγ activates the phosphatidylinositol 3-kinase (PI3K), which leads to GP IIb/IIIa receptor activation through activation of a serine-threonine protein kinase B (PKB/Akt) and of Rap1b GTP binding proteins. Prostaglandin E_1 (PGE_1) activates AC, which increases cAMP levels and status of VASP-P. Solid arrows = activation; dotted arrows = inhibition.

Recently more potent ADP-receptor antagonists like ticagrelor and the third-generation of thienopyridines, as prasugrel are being investigated or under approval. Both of these substances have a demonstrated higher efficacy in acute coronary syndrome patients. (33;34) However there are mostly pharmacodynamic studies that tested the possible biological benefit of switching to prasugrel or to ticagrelor in patient with low-response to clopidogrel. (35;36)

2.4 Interindividual differences in response to anti-platelet therapy

Despite the high efficacy, safety and low cost, patients do not benefit equally from standard dose anti-platelet therapy. In case of aspirin in a proportion of patients, classified as „aspirin-resistant" or „non-responder", aspirin dose not inhibit platelet activation and aggregation.

Although these subjects can be separated from good responders with laboratory assessments, the exact prevalence of aspirin resistance in patients suffering from stable coronary artery disease remains unclear. (1) It ranges from 5% to 65%; this may be due to differences in studied populations, lack of formal definition of aspirin response, and use of non-standardized diagnostic methods. (1;2)

As DAPT with aspirin and clopidogrel became extensively used, so did clopidogrel poor responsiveness / resistance emerge, affecting approximately 20-30% of the patients. (3-5) Non-responsiveness to clopidogrel therapy manifest with an increased risk of ischemic events, especially stent thrombosis after coronary intervention. (37-44) During the last decade many trials have been focused on the large interindividual differences in response to a fix-dose clopidogrel. (3-5;45)

As the active metabolite formation is influenced by genetic, clinical and pharmacological factors, the development of high on-clopidogrel platelet reactivity (HPR) is a multifactorial process. (27) As recently evidenced, low compliance is also a remarkable reason for measuring HPR in the patient. (46) Up to now, numerous *in vitro* or *ex vivo* laboratory assays have been developed to monitor on-clopidogrel platelet reactivity.(11) Using these assays, accumulating number of observational studies have found that patients with HPR have higher risk for recurrent ischemic events, including myocardial infarction and stent thrombosis. (39-41;44;47-62) However, routine platelet function testing is not yet recommended. (63) First, this is due to the somewhat arbitrary-used and non-standardized definitions for HPR. Second, the prognostic significance of HPR after PCI is unclear as prospective, adequately-powered clinical trials are lacking. Third, there is no consensus on the ideal platelet function assay to monitor on-clopidogrel platelet reactivity. (21)

The historical gold-standard that has been shown to predict clinical outcomes after PCI is the ADP stimulated platelet aggregation with Light Transmission Aggregometry (LTA). (62)

Though there has been no consensus regarding the type and concentration of agonist and the optimal estimate used to express $P2Y_{12}$ receptor inhibition. The majority of the laboratories are using the peak value (Agg_{max}) of the ADP-stimulated aggregation curve that is achieved early after agonist addition. However, LTA is not fully $P2Y_{12}$-specific, as ADP also binds to the P2Y1 receptor. Thereby, some authors prefer to use the late aggregation (Agg_{late}) value supposing that aggregation measured at 5-6 minutes after ADP stimulation might better represent $P2Y_{12}$ receptor inhibition without the influence of P2Y1-activity. (39;64) However, the superiority of Agg_{late} over other aggregometry parameters has not yet been proved by an independent, $P2Y_{12}$-specific assay, such as VASP-PRI or VerifyNow.

2.5 Genetic variations of ABCB1, Cytochrome P450 2C19 (CYP2C19), and Paraoxonase 1 (PON1) genes

The absorption of clopidogrel in the gastrointestinal system is mediated by an ATP-dependent efflux transporter, P-glycoprotein (P-gp), encoded by the multidrug resistance 1 gene (mdr1, ABCB1). (65) The function of the transporter is to prevent the body from environmental toxins and drugs by limiting their absorption from the lumen of the gut or increasing their biliary and urinary excretion. (66) The most widely studied genetic variants of the ABCB1 gene, that could have causal link with altered P-gp function, are the two partly linked polymorphisms, the 2677G→T/A and 3435C→T, located on exons 21 and 26.

Results of an MDR1 genetic study, focusing on ABCB1 C3435T and G2677T/A SNPs in the Hungarian population are in line with data of other studies about the Caucasian population. Occurrence of the 3435C/C genotype is approximately 17.7%, 3435T/T is around 32.4%, while 3435C/T is around 49.9%. (67) Results of the population genetic study also supported the previously observed significant link between the wild type-wild type sequence (3435C/C-2677G/G) and variant-variant sequence (3435T/T-2677T/T/A) in position C3435T and

G2677T/A. (68) Nearly 60% of the Hungarian population is either wild type or variant homozygotes or heterozygotes simultaneously in both SNPs. (67)

However the effect of single C3435T and G2677T/A SNPs or different haplotypes on ABCB1 expression and function still remains unclear.

Findings of different studies highlighting on ABCB1 SNPs' effects are confusing. Taubert et al found that after 600 mg loading dose of clopidogrel 3435T/T carriers have higher level of platelet aggregation, lower level of absorbed clopidogrel and active metabolite. (65) This observation was later confirmed clopidogrel in a nationwide French registry (FAST-MI), and in the genetic substudy of the TRITON TIMI38 trial showing a 72% relative increase in the rate of death, myocardial infarction or stroke in patients treated with clopidogrel. (69;70) On the contrary, there were no significant differences in clinical outcomes regarding ABCB1 genotypes among clopidogrel-treated patients in the PLATO genetic substudy or in a genome-wide association study of Gurbel et al. (71;72) Contrary in the PLATO analysis 3435C/C carriers showed numerically higher risk to cardiovascular death, MI or stroke compared to C/T or T/T carriers. Importantly, these results are biologically plausible, as the higher expression of P-gp (3435C/C genotype) might be associated with a more efficient efflux of clopidogrel from enterocytes into the intestinal lumen. (71) While Mega and colleagues found no interaction with platelet inhibition or prognosis, in accordance with the 2677G→T/A SNP. (73)

Among different isoenzymes, CYP2C19 has been proven to modulate clopidogrel pharmacokinetics and pharmacodynamics in healthy volunteers as well as in patients. (74-76) The CYP2C19 gene has more than 35 allelic variants (http://www.cypalleles.ki.se/cyp2c19.htm). (77-79) Population can be divided to extensive metabolizer (EM), intermedier metabolizer (IM), poor metabolizer (PM) and ultrarapid metabolizer (UM) groups according to their genotypes. (Table 5.3.2) The main non-functional

alleles of CYP2C19*2 and *3* account for 87% of the PM predicted phenotype in Caucasians and 98% in Orientals. (80) Additionally, the novel gain-of-function (GOF) allelic variant CYP2C19*17, which encodes the ultrarapid enzyme form, occurs around 36% in Caucasians. (81;82)

Acknowledging the clinical importance of CYP2C19 allelic variants, the FDA has issued a boxed warning stating that poor metabolizers do not profit to the same extent as wild-type allele carriers from clopidogrel treatment. (83) Beside the importance of LOF alleles, prognostic value of the GOF genetic variant CYP2C19*17 is unclear: some data suggest an increased risk of bleeding among individuals carrying this allelic variant. (71;82) Interactions of the different functional allelic variants also must be taken into consideration. Prior work of Sibbing and colleagues, observed a gene-dose effect: those harboring two GOF alleles have the lowest, while patients carrying two loss-of-function alleles (*2, *3) have the highest degree of post-clopidogrel platelet aggregation and $P2Y_{12}$-receptor activity. (84) At the same time previous studies indicate that only 3.7% to 12% of the interindividual variability is explained by CYP2C19 genetic variants. (72;85)

The main allelic variant of the paraoxonase 1 (PON1) gene, determining the enzyme activity, is the single glutamine (Q) – arginine (R) amino acid substitution at position 192 (Q192R). Effects of this SNP on CAD and on clopidogrel efficacy however remain conflicting. On one hand the PON1 192R/R isoform is found to be less effective at hydrolyzing lipid peroxides than Q/Q isoform, hence PON1-R allele is positively associated with CAD, while Q allele is protective against atherosclerosis. (86) On the other hand PON1 is also known to be a factor in the second step of the biotransformation of clopidogrel; i.e. the conversion of 2-oxo-clopidogrel to the active thiol derivate. According to a study of Boumann and colleagues individuals carrying the 192Q/Q genotype had lower plasma active metabolite concentrations, lower inhibition of platelet aggregation and higher risk for adverse ischemic events after PCI.

(28) Since, study by Sibbing and colleagues confuted the influence of PON-1 Q→R SNP on platelet reactivity and clinical outcome. (87)

3. Aims

According to the recent guidelines of the European Society of Cardiology, the American College of Cardiology and the American Heart Association, for the prevention of recurrent thrombotic events after percutaneous coronary intervention (PCI), dual anti-platelet therapy with aspirin and clopidogrel is recommended. (29;30) Considering the findings of previous reports in the subject, emphasizing great interindividual diffrences in response to fix-dose clopidogrel therapy, as well as the absence of standardized, comparable platelet-function assays and well defined cut off values, in our study we pursued the following aims:

1. To perform systematic review over the available literature highlighting on methodical heterogeneity, comparing results and predictive value of different anti-platelet function assays on defining platelet reactivity and clinical outcome.

2. To compare utility and reliability of the historical gold-standard light transmission aggregometry (LTA) with the flow cytometer based vasodilator-stimulated phosphoprotein phosphorilation (VASP-PRI) assay. We designed a clinical study to:

2.1 investigate the most optimal and predictive platelet aggregation parameter (Agg_{max}, Agg_{late}, AUC or disAggregation) measuring platelet reactivity and predicting clinical outcome;

2.2 to compare LTA estimates in determining the potency of $P2Y_{12}$ receptor inhibition to VASP-PRI assay.

3. To investigate the influence of main genetic variants affecting clopidogrel anti-platelet effect. A clinical study was designed to:

3.1 determine the impact of main genetic variants of cytochrome P450 2C19 (CYP2C19) gene as CYP2C19*2 and CYP2C19*3 loss-of-function and CYP2C19*17 gain-of-function variants on clopidogrel efficacy and clinical outcome, among low-risk, stable angina patients after elective PCI;

3.2 determine the impact of the main genetic variants of multidrug resistance-1 gene (ABCB1, mdr1) as C3435T and G2677T/A on clopidogrel efficacy and clinical outcome, among low-risk, stable angina patients after elective PCI;

3.3 determine the impact of the main genetic variant of paraoxonase-1 (PON1) gene as Q192R on clopidogrel efficacy and clinical outcome among low-risk, stable angina patients after elective PCI.

4. Methods

4.1 Laboratory methods to assess anti-platelet efficacy

4.1.1 Light Transmission Aggregometry (LTA)

The first aggregation test was introduced into laboratory practice in the late ’60-ies. It used platelet-rich plasma (PRP), and aggregation was detected based on an optical method. The assessment of platelet function using whole blood (WB) aggregation by an impedance method followed up nearly 20 years later.

Platelet aggregation testing measures the ability of various agonists, such as ADP, to platelets to induce *in vitro* activation and platelet-to-platelet activation. The method of Light Transmission Aggregometry (LTA) was developed to fit the requirement of routine testing and it had become the gold-standard of measuring platelet aggregation in subjects with platelet function disorders and in patients receiving anti-platelet therapy. The method is relatively cheap, widely used and well accepted; enables monitoring anti-platelet efficacy selectively with specific agonists. At the same time great disadvantage of the method is that, it is poorly standardized resulting in difficulties to compare results and generalize consequences obtained with this method. Furthermore, analysis is time-consuming, non-automated and requires trained staff for sample preparation and measurements. In our studies CARAT TX4 four-channel light transmission aggregometer (Carat Diagnostics, Hungary) was used for platelet function assessments. Calibration for aggregation measurements were established using light transmission percentage through platelet rich plasma (0% transmission) and platelet poor plasma (100% transmission). Platelet rich plasma (PRP) was obtained from the supernatant of plasma being centrifuged at 1600 rpm for 4 minutes. Further centrifuging at 4000 rpm for 10 minutes resulted in platelet poor plasma (PPP) in the supernatant. Platelet

count in the PRP varied from 250 to $350x10^9$/L. No adjustment of platelet count with PPP was performed, as it has been demonstrated that the adjustment of PRP with PPP decreases reliability of the measurement. (88)

The assessment required 10ml sodium-citrate (3.8%) anti-coagulated blood from each patient. All samples were processed within two hours. For the calibration of the aggregometer, PPP was used to set 100%, while PRP to 0% light transmission. After sample separation to PRP and PPP fractions, platelet-specific agonists were added into PRP to stimulate platelet aggregation with a continuous magnetic stirring at 37°C. In anti-platelet free subjects, activation of resting platelets resulted 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 described with the maximal platelet aggregation value (Agg_{max}) of the registered optical curve, while other parameters (late aggregation [Agg_{late}], steepness of slope, area under curve [AUC] and disAggregation [disAgg] may also be determined. (Figure 2, 3)

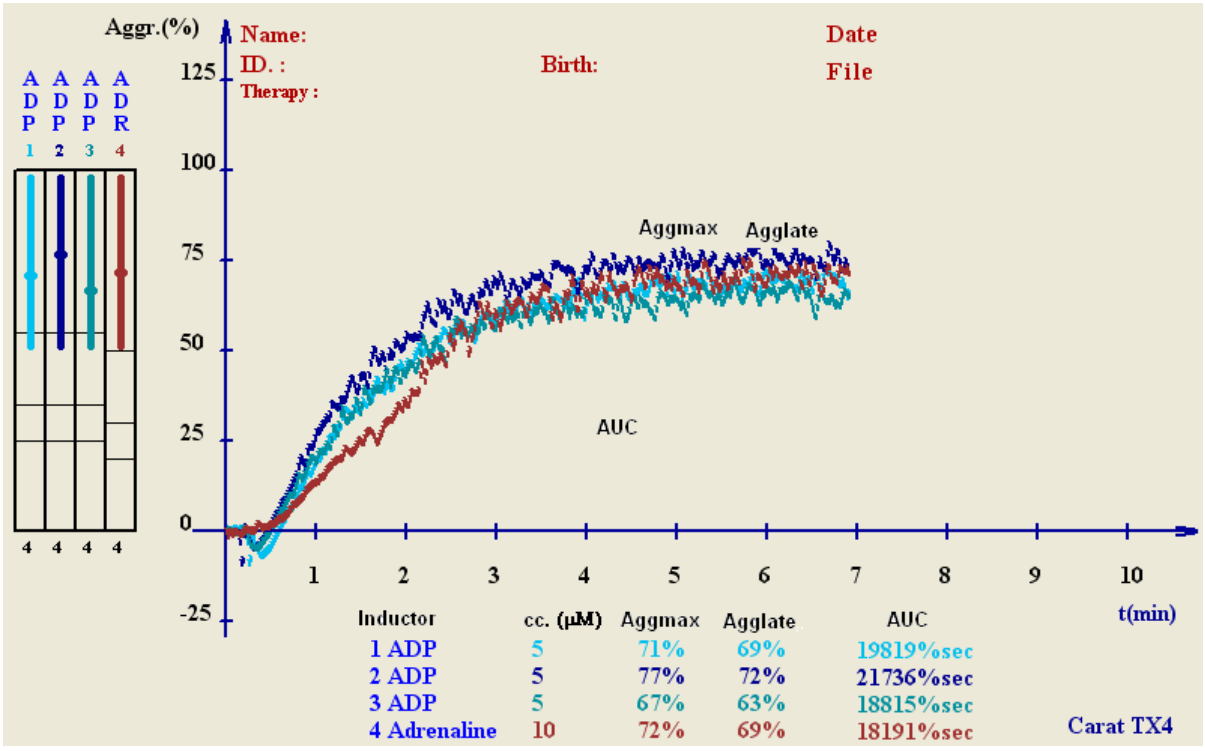


Figure 2. Light transmission assessment with ADP 5 µM (performed in triplicate) and adrenaline 10 µM in a patient not exposed to anti-platelet therapy. Agg_{max}: maximal aggregation, Agg_{late}: 6-minute late aggregation, AUC: area under the curve. AUC is calculated as the sum of the actual aggregation values in every second from agonist addition until 6 minutes; devided by 100. DisAggregation was defined according to $(Agg_{max}- Agg_{late})/Agg_{max}$ x100.

Efficient anti-platelet therapy inhibits platelet activation, limiting the formation of platelet aggregates and decreasing the peak value of the aggregation curve. Likewise, high Agg_{max} values are typical for untreated subjects and low responder patients, while low Agg_{max} reflects effective platelet inhibition. (Figure 2)

As anti-platelet 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 2, 3)

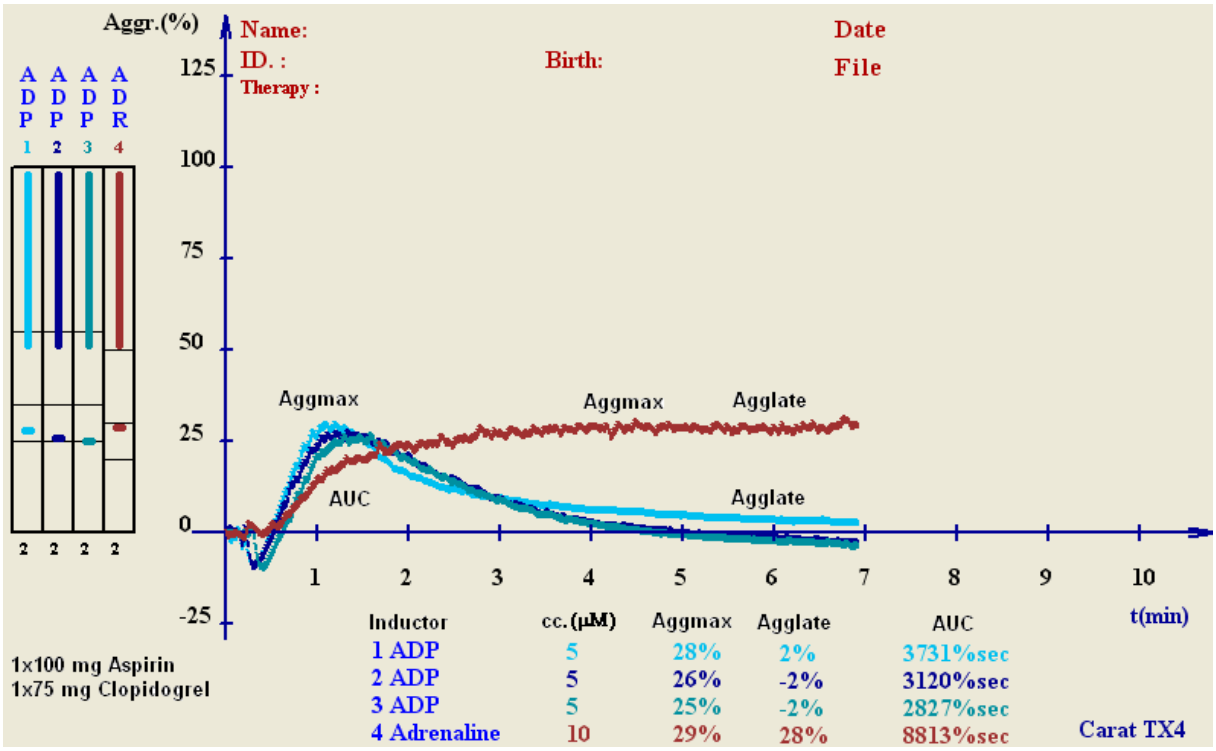


Figure 3. Light transmission assessment with ADP 5 µM and adrenaline 10 µM in a patient with good response to aspirin and clopidogrel therapy. Agg_{max}: maximal aggregation, Agg_{late}: 6-minute late aggregation, AUC: area under the curve. AUC is calculated as the sum of the actual aggregation values in every second from agonist addition until 6 minutes; devided by 100. Disaggregation was defined according to $(Agg_{max}- Agg_{late})/Agg_{max}$ x100.

4.1.2 Flow cytometric assessment of vasodilator stimualted phosphoprotein phosphorylation assay (VASP-PRI)

LTA measures platelet aggregation, evaluating the total final effect of the aggregation cascade. Flow cytometry, on the other hand, using fluorescent monoclonal antibodies and probes, gives the unique opportunity to measure specific aspects of platelet activation on an individual cell level. Platelets in whole blood are incubated with fluorescent probes. Platelets in the suspension are drawn into the flow chamber in the flow cytometer and through the beam of a laser. Activation of the fluorophore occurs, and emitted light scatter properties and fluorescence are detected. In the vasodilator stimulated phosphoprotein phosphorylation assay (VASP-PRI), intracellular signaling followed by $P2Y_{12}$ activation is assessed when VASP in its phosphorylated state is labeled by immunofluorescence using a specific monoclonal antibody.

Vasodilator-stimulated phosphoprotein (VASP) is non-phosphorylated in the basal state and the phosphorylation of VASP is regulated by the cAMP cascade. Prostaglandin E1 activates the cascade whereas it is inhibited by ADP through the $P2Y_{12}$ receptor. (Figure 4)

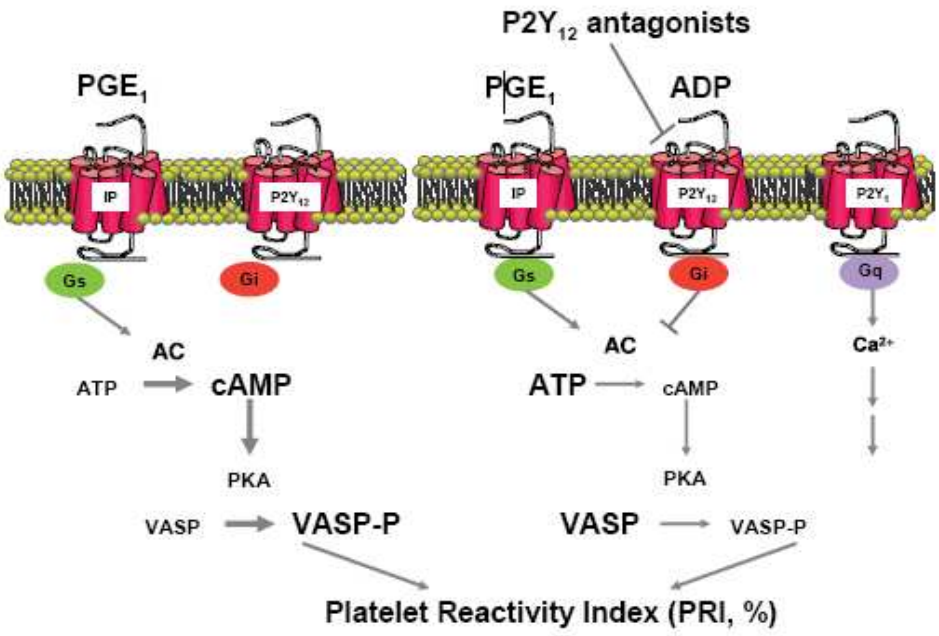


Figure 4. Vasodilator-stimulated phophoprotein phosphorylation.

(Platelet Inhibition in Coronary Artery Disease-Mechanisms and Clinical Importance. Varenhorst C. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine)

The VASP assay was performed using a commercially available method according to the manufacturer's specifications (Biocytex Platelet VASP kit, Marseille, FR). In brief, citrated whole blood was incubated with prostaglandin E1 (PGE1) and PGE1 plus ADP for 10 min and fixed with paraformaldehyde, after which the platelets were permeabilized with nonionic detergent. The cells were labeled with a primary monoclonal antibody against serine 239-phosphorylated VASP, followed by a secondary fluorescein isothiocyanate-conjugated polyclonal goat anti-mouse antibody. The platelet population was identified with an anti-CD61 phycoerythrin-labeled antibody. Mouse monoclonal antibody was used as negative isotypic control. Mean fluorescence intensities (MFI) were recorded on a Beckmann Coulter flow cytometer gating 10000 platelets for every measurement. Corrected mean fluorescence intensities (MFIc) were obtained after subtraction of the MFI value with the isotypic control.

Platelet reactivity index (PRI) was calculated from the corrected mean fluorescence intensities (MFIc) according to the following equation: (Figure 5)

$$MFIc_{PGE1} = MFI_{PGE1} - MFI_{(isotypic\ control)}$$

$$MFIc_{(PGE1+ADP)} = MFI_{(PGE1+ADP)} - MFI_{(isotypic\ control)}$$

$$PRI = \frac{(MFIc_{PGE1} - MFIc_{(PGE1+ADP)})}{MFIc_{PGE1}} x100$$

Figure 5. Calculation of platelet reactivity index.

4.2 Genetic analysis of the functionally relevant ABCB1, CYPC19 and PON1 allelic variants

4.2.1 Blood sampling

For the genetic analysis of ABCB1, PON-1 and CYP2C19 loci, genomic DNA was isolated from 200 µL EDTA-anticoagulated blood samples using the High Pure PCR Template Preparation Kit (Roche Applied Science). All of the genotyping procedures were performed using the LightCycler 2.0 Real-Time PCR System (Roche Applied Science) Melting Curve Analyzer program. CYP2C19*2 (681G→A, rs4244285) and CYP2C19*3 (636G→A, rs4986893) genetic variants were determined by LightMix Kit human CYP2C19*2 and CYP2C19*3 (Roche Applied Science). In case of CYP2C19*17 allele (806C→T, rs12248560) and ABCB1 SNPs (3435C→T, rs1045642 and 2677G→T/A rs2032582), sequence specific primers and fluorescent labeled probes (TIB MOLBIOL GmBH, Berlin) were used. For the identification of PON-1 192Q→R (rs662) SNP, the allele specific amplification method, described by Pocsai et al, was used. (70) The predicted metabolizer

(CYP2C19 and PON-1) and expresser (ABCB1) phenotypes were assessed based on the determined genotypes according to the definition of previous studies.

4.3 Real Time PCR (RT PCR)

The LightCycler RT PCR is a fluorescent based system using sequence specific, fluorescent dye labeled oligonucleotid probes. For the identification of certain gene's genotype, melting curve profiles are used. To analyze the sample melting temperature profile, the fluorescence of the sample is being monitored while the LightCycler temperature is steadily increased. As the temperature increases, sample fluorescence decreases. For HybProbe system, this is due to the separation of target-probe hybrids resulting in the separation of the dye molecules and a consequent drop in fluorescence. The melting temperature (Tm) of a sample is defined as the point at which half the probes (or dye) have melted off the DNA. Tm can vary over a wide range, depending on the length and sequence of the strand. Even single-base differences in the DNA can result in melting temperature shifts. According to such character of the Tm, melting temperature profile can be used to identify and genotype DNA products. The analysis displays a melting curve chart of sample fluorescene versus temperature. (Figure 6, 7)

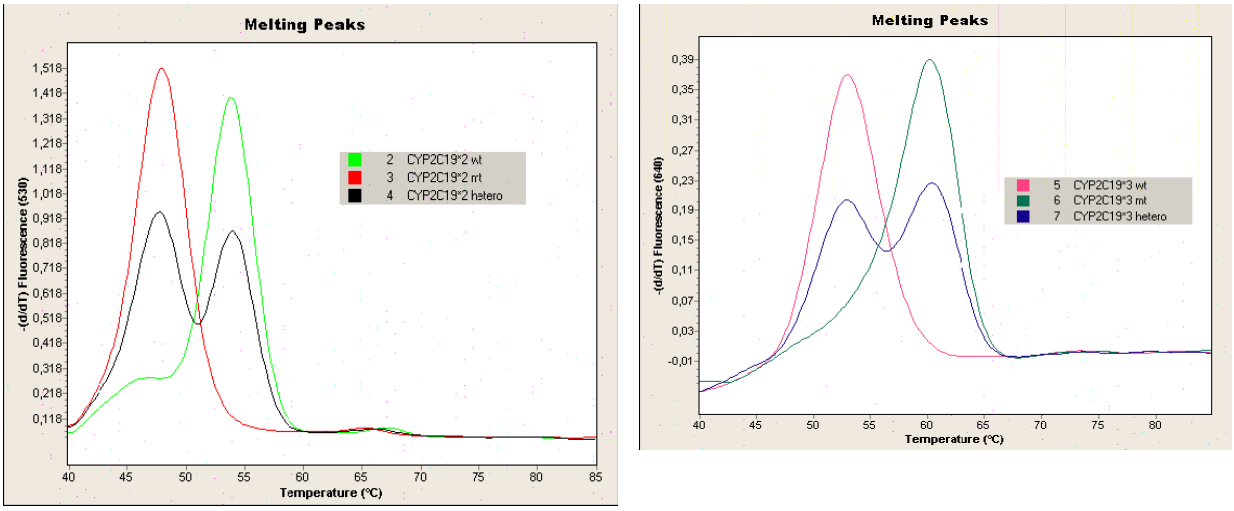


Figure 6. Melting curve analysis of the CYP2C19 *2 and *3 genetic variants. The CYP2C19*1 wildtype genotype exhibited a Tm of 54.1 ºC in channel 530nm and a Tm of 52.9 ºC in channel 640nm. The allele variant CYP2C19*2 exhibited a Tm of 47.8 ºC in channel 530nm and the allele variant CYP2C19*3 exhibited a Tm of 60.5 ºC in channel 640nm.

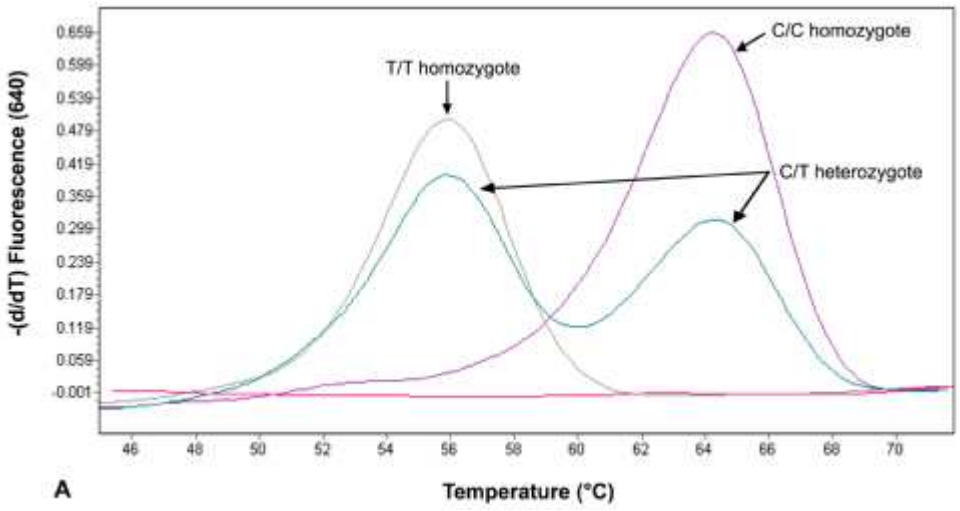


Figure 7. Melting curve analysis of the ABCB1 C3435T genetic variant. The ABCB1 C3435C wildtype genotype exhibited a Tm of 64 ºC in channel 640nm. The allele variant ABCB1 T3435T exhibited a Tm of 56 ºC in channel 640nm and the allele variant ABCB1 C3435T exhibited a Tms of 56 ºC and 64 ºC in channel 640nm.

Sequencies of the specific primers and probes, are depicted in Table 1.

ABCB1 3435T>C; rs1045642 Forward primer: 5’-TGTTTTCAGCTGCTTGATGG-3’ Reverse primer: 5’-AAGGCATGTATGTTGGCCTC-3’ Fluoropro: 5’-GACAACAGCCGGGTGGTGTCA-Fluo-3’ LCpro: 5’-LCRed-640-GGAAGAGATCGTGAGGGCAG-Pho-3’
ABCB1 2677G>T/A; rs2032582 Forward primer: 5’-GTCCAAGAACTGGCTTTG-3’ Reverse primer: 5’-TGGCAACTAACACTGTTAC-3’ Fluoropro: 5’-ACCTTCCCAGAACCTTCTAG-Fluo-3’ LCpro: 5’-LCRed-640-CTTTCTTATCTTTCAGTGCTTGTCCAGAC-Pho-3’
CYP2C19*17 806C>T; rs12248560 Forward primer: 5’-CAGAATAACTAATGTTTGGAAGTTG-3’ Reverse primer: 5’-AGAACTGGGATTTGAGCTG-3’ Fluoropro: 5’-TGTTCTCAAAGCATCTCTGATGTAAGAGATAATG-Fluo-3’ LCpro: 5’-LCRed-640-CCACGATGGGCATCAGAAGACCT-Pho-3’
PON1 192Q192R; rs662 Forward primer: 5’-TATTGTTGCTGTGGGACCTGAG-3’ Reverse primer: 5’-CCTTCTGCCACCACTCGAAC-3’ Fluoropro: 5’-CCCCTACTTACAATCCTGGGAGAT-Fluo-3’ LCpro: 5’-LCRed-705-ATTTGGGTTTAGCGTGGTCGTATGTTG-Pho-3’

Table 1. Sequencies of the specific primers and probes used for the genetic analyses.

4.4 Study design and patient population

During the systematic review and meta-analysis PubMed and Central databases were searched for relevant articles published between January 2003 and February 2010. Search key words included various combinations of "clopidogrel" with the following terms: resistance, platelet reactivity, outcome and prognostic. No language restriction was used. We also searched the reference lists of relevant studies and reviews, editorials, and letters. In case of incomplete reporting, individual authors were contacted.

4.4.1 Selection criteria

We selected all relevant studies which met the following inclusion criteria:

(1) Studies that recruited patients receiving aspirin and clopidogrel therapy after percutaneous coronary intervention (PCI);

(2) Reported an intention-to-treat analysis on the clinical impact of HPR measured by an ADP-specific platelet function assay. No case-control studies were accepted. The accepted assays included ADP-stimulated Light Transmission Aggregometry (LTA_{ADP}), flow cytometric assessment of vasodilator-stimulated phosphoprotein phosphorylation (VASP), the VerifyNow device using $P2Y_{12}$ cartridge ($VerifyNOW_{P2Y12)}$, and multiple electrode aggregometry with ADP stimuli (MEA_{ADP}). In case of studies using more than one assay, two assays with the highest predictive values were selected.(51;61;62)

(3) All studies that measured platelet aggregation values after a loading dose or on maintenance phase of clopidogrel therapy were eligible. Examinations that assessed responsiveness to clopidogrel, i.e. a difference between baseline and post-treatment platelet reactivity (IPA), were excluded from the analysis.

The primary clinical outcomes of interest, evaluated at the longest available follow-up (during that patients were on clopidogrel treatment) were (a) cardiovascular (CV) death, (b)

definite/probable stent thrombosis (ST), (c) non-fatal myocardial infarction (MI; Type 1, 4a, 4b) and (d) a composite endpoint of the reported ischemic events (CIE) that included CV death, MI, ischemic stroke, unplanned repeat revascularisation or rehospitalisation for ACS. Those studies that evaluated solely peri-procedural MI (Type 4b) were excluded.

Along in the clinical studies (LTA-VASP-PRI comparison and genetical studies) clopidogrel-naïve stable angina patients in whom elective percutaneous coronary interventions (PCI) were performed, had been recruited.

Exclusion criteria were acute coronary syndrome (ACS), 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 x109/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. Twelve to 18 hours after receiving the clopidogrel loading dose, blood was drawn from each patient from peripheral vein for LTA, VASP assessments to measure the extent of on-clopidogrel platelet reactivity and for genetic analysis. Based on the results, patients with HPR were randomized in a 1:1 ratio to receive either 75 mg or 150 mg clopidogrel for 4 weeks, while those with normal platelet reactivity continued 75 mg clopidogrel. After 28 days, all patients returned to 75 mg maintenance dose of clopidogrel until one year.

During follow-up, clinical events, such as cardiovascular (CV) death, non-fatal myocardial infarction (MI), stent thrombosis (using the ARC criteria), stroke and target vessel revascularizations (TVR) were recorded until one year. The evaluated primary composite endpoint of the study was the occurrence of CV death, non-fatal MI or unplanned TVR at one year. Bleeding events were only counted for safety monitoring, not with intent for group comparison.

4.5 Definition of high on-clopidogrel platelet reactivity (HPR) in our clinical studies

High platelet reactivity was defined as a VASP-PRI value ≥50%. This cutoff was selected on the basis of previous results showing a significantly elevated ischemic risk for patients above this threshold. (50;88)

The definition for HPR in optical aggregometry was originally an Agg_{max} value ≥34%. a ROC based cut off value originating from our previous publication with the same laboratory method and comparable patient population; (52) however, a recently published consensus paper recommended 46% maximal aggregation value in case of 5 μM ADP-stimulated LTA assessment. Although the cut off used in our studies were also based on ROC curve analysis, following the recommendations of the consensus paper, we defined HPR as an Agg_{max} value > 46%. (50;51)

4.6 Statistical Analysis

Statistical analysis of the systematic review and meta-analysis was performed using the Review Manager 5.0.22 freeware package maintained by the Cochrane Collaboration. (Review Manager [RevMan]. Version 5.0.22 Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2008.) Hazard ratios (HR) and odds ratios (OR) adjusted for the relevant clinical parameters, from individual studies, were pooled with the random-effect model via generic-inverse variance-weighting. If the adjusted relative risk was not reported, the odd ratios were calculated from the reported event frequencies. Heterogeneity was quantified with a Chi^2 heterogeneity statistic and by means of I^2. Group comparisons in case

of the prevalence of HPR were done with the Kruskal-Wallis test. A p value < than 0.05 was considered significant.

Statistical analysis in the clinical studies was done by the SPSSv11.0 (SPSS Inc. Chicago, Illinois) and Graphpad Prism 5.0 trial (Graphpad Software Inc). Spearmann'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. 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 cut off points were determined according to the threshold with the highest summation of sensitivity and specificity.

Agreement among assays to identify patients with normal or high platelet reactivity was assessed through the κ statistic. A p value <0.05 was considered statistically significant in all analysis.

In the genetic trial, differences between treatment groups were assessed with the Chi-square test for categorical variables. Unpaired t-tests were used for comparison of normally distributed, continuous variables between two different groups. Non-normally distributed variables were analyzed with the Mann-Whitney test between two groups. In case of more than two groups, the results were compared with the Kruskall-Wallis non-parametric test. Cox proportional hazard models were used to compare event-free survival in the treatment arms. In these models, univariable and multivariable analyses were used to calculate unadjusted and adjusted hazard ratios (HR) with 95% confidence intervals (CI). All clinical, procedural and laboratory variables (Table 5.3.1) that showed a p value ≤ 0.30 in the univariate analysis were entered in the multivariate model executed by forward conditional modeling. Beyond these covariates, know predictors of HPR (diabetes, gender, age, BMI, PPI administration) were also entered in the multivariate model. Survival differences between groups were demonstrated with Kaplan-Meier hazard curves and were compared with the Mantel-Cox log rank test. Linear regression models were used to determine the proportion (R^2) of variability in platelet reactivity explained by different genotypes. Univariate and multivariate binary logistic regression models were used to calculate odds ratios for the risk of HPR among genotypes. The predictive power of different genotypes on HPR was determined by a receiver operating characteristic curve analyses. The positive (PPV) and negative predictive values (NPV) for group distinction were determined as follows: PPV (%) = true positives / (true positives + false positives) x 100; NPV (%) = true negatives / (true negatives + false negatives) x 100.

5. Results

5.1 Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: Systematic review and meta-analysis

5.1.1 Study selection of the systematic review and meta-analysis

Our search resulted in 1801 citations. These included reviews and articles that did not meet our inclusion criteria. After careful title and abstract evaluation, 31 potentially appropriate studies were found. Of the 31 studies, 20 articles including a total number of 9187 patients were selected for full text analysis and data extraction, after excluding case-control studies. (39-41;44;47-49;51-63) Out of these, 13 studies measured platelet reactivity with $LTA_{ADP,}$ 5 with the VerifyNowP2Y12 assay 3 with VASP phosphorylation, and 2 with the MEA_{ADP} device. Major characteristics of included studies are depicted in Table 5.1.1.

Investigator (Publication year)	Design, patient profile	Number of patients	Laboratory methods	Selected cut off for HPR	HPR (%)	Clopidogrel LD / MD	End point	Follow-up	Quality score[†]
Cuisset (2006) [47]	RCT, NSTEMI	146[*]	LTA: 10µmol ADP, Agg_{max}	Agg_{max}≥70%	15.1	600 / 75	CIE	1 month	NA
Cuisset (2006) [40]	Prospective, NSTEMI	106	LTA: 10µmol ADP, Agg_{max}	Highest quartile (Agg_{max}≥70%)	21.7	300 / 75	CIE	1 month	9
Geisler (2006) [41]	Prospective, all-comer	363	LTA: 20µmol ADP, Agg_{max}	Agg_{max}≥70%	6.1	600 / 75	D / MI / CIE	3 months	9
Hochholczer (2006) [39]	Prospective, stable angina	802	LTA: 5µmol ADP, Agg_{late}	Above the median (Agg_{late}≥15%)	49.3	600 / 75	D / MI / CIE	1 month	9
Angiolillo (2007) [48]	Prospective, stable angina with DM	173	LTA: 20µmol ADP, Agg_{max}	Highest quartile (Agg_{max}≥62%)	26.0	0 / 75	CIE	2 years	9
Bliden (2007) [49]	Prospective stable angina	100	LTA: 5µmol ADP, Agg_{max}	Agg_{max}≥50%	22.0	0 / 75	MI / CIE	1 year	9
Bonello (2007) [89]	Prospective, stable/unstable angina	144	VASP: PRI	Highest quintile (PRI >47%)	79.9	300 / 75	D / CIE	6 months	9
Buonamici (2007) [44]	Prospective, all-comer	804	LTA: 10µmol ADP, Agg_{max}	Agg_{max}≥70%	13.1	600 / 75	D / ST	6 months	9
Frere (2007) [51]	Prospective, NSTEMI	195	VASP: PRI LTA: 10µmol ADP, Agg_{max}	PRI≥53% Agg_{max}≥70% (ROC-defined)	54.4 29.8	600 / 75	CIE	1 month	9
Aradi (2008) [52]	Prospective, stable/unstable angina	108[§]	LTA: 5µmol ADP, Agg_{max}	Patients above the median (Agg_{max}>33%)	51.9	300 / 75	MI / ST / CIE	10 months	9
Patti (2008) [53]	Prospective, all-comer	160	VerifyNow	Highest quartile (>240 PRU)	25.0	600 / 75	CIE	1 month	9
Price (2008) [54]	Prospective, all-comer	317[#]	VerifyNow	≥235 PRU (ROC-defined)	34.1	600 / 75	D / MI / ST / CIE	6 months	9
Castro (2009) [55]	Prospective, NSTEMI	161	VerifyNow	>175 PRU (ROC-defined)	39.8	300 / 75	CIE	1 year	9
Cuisset (2009) [56]	Prospective, NSTEMI	598	LTA 10 µmol ADP, Agg_{max} VASP, PRI	Agg_{max} > 67% (ROC-defined) PRI: not reported	30.9	600 / 75	ST	1 month	9

Investigator (Publication year)	Design, patient profile	Number of patients	Laboratory methods	Selected cut off for HPR	HPR (%)	Clopidogrel LD / MD	End point	Follow-up	Quality score†
continued									
Geisler (2009)[57]	Prospective, all-comer	1019	LTA: 20 µmol ADP, Agg_{late}	Patients of the highest quartile (>42.5%)	32.3	600 / 75	D / MI ST / CIE	3 months	9
Marcucci (2009) [58]	Prospective, ACS	683	VerifyNow	≥240 PRU (ROC-defined)	32.1	600 / 75	D / MI / CIE	1 year	9
Migliorini (2009)[69]	Prospective, all-comer (LM)	215	LTA: 10 µmol ADP, Agg_{max}	Agg_{max} > 70%	18.6	600 / 75 or 150	D / MI ST	3 years	9
Sibbing (2009) [60]	Prospective, all-comer	1608	MEA: AUC	Highest quintile (>416 AU)	20.1	600 / 75	D / MI ST	1 month	9
Siller-Matula (2009)[61]	Prospective, all-comer	416	MEA: AUC VASP: PRI	AUC > 54 U PRI > 23 % (ROC-defined)	13.5 63.0	600 or 0 / 75	ST	6 months	9
Breet (2010)[62]	Prospective, stable angina	1069	LTA: 5 µmol LTA: 20 µmol ADP, Agg_{max} VerifyNow	Agg_{max}≥42.9% Agg_{max}≥64.5% ≥236 PRU (ROC-defined)	42.4 37.3 38.6	300/75 or 600/75 or 0/75	D / MI ST /CIE	1 year	9

Abbreviations: ADP: adenosine 5'-diphosphate; Agg_{max}: maximal aggregation; Agg_{late}: late aggregation; AU: arbitrary unit; AUC: area under curve; CIE: composite ischemic events; D: cardiovascular death; DM: diabetes mellitus; LD: loading dose; LM: left main stenting; LTA: Light Transmission Aggregometry; MEA: Multiple Electrode Aggregometry; MI: non-fatal myocardial infarction; MD: maintenance dose; NA: not applicable; NSTEMI: non-ST-segment elevation MI; PRI: platelet reactivity index; PRU: platelet reaction unit; RCT: randomized controlled trial; ST: stent thrombosis; VASP: vasodilator-stimulated phosphoprotein phosphorylation analysis; †: New Castle Ottawa score: quality assessment of observational studies; this scoring is not applicable for randomized, controlled trials; *: data from the 600-mg loaded group, §: data from clopidogrel-treated patients, #: patients on clopidogrel for at least 6 months.

Table 5.1.1. Detailed description of the selected studies.

5.1.2 Prevalence of HPR

In the 20 studies, including 9,187 patients, the rate of HPR showed large heterogeneity with a mean prevalence of 32.3% (95% CI for mean 25.9-40.5, range 6.0679.86). Finding possible determinants of the observed heterogeneity, the prevalence of HPR was analyzed according to the following groupings: type of platelet function device, the selected platelet reactivity cut off, the amount of clopidogrel loading dose, the time of assessment from loading/last clopidogrel dose, and the proportion of acute coronary syndrome (ACS) patients in each group. (Table 5.1.2) Among the recruited studies, the selected platelet reactivity cut off and the type of the platelet function device interacted significantly with the prevalence of HPR (Kruskal-Wallis test P = .04 and P = .02, respectively). (Table 5.1.2) The selected cut off was in strong, inverse correlation with the rate of HPR. (Figure 5.1.1) The highest rates of HPR were measured by the $P2Y_{12}$-sensitive VASP-PRI assay.

	Mean, % (95% CI)	P
Overall	33.2 (25.9 – 40.5)	
Platelet Function Method		
MEA_{ADP}:	16.8 (-25.1 – 58.7)	***0.02***
LTA_{ADP}:	28.3 (20.5 – 36.1)	
$VerifyNOW_{P2Y12}$:	33.9 (26.6 – 41.2)	
VASP:	65.8 (33.5 – 98.0)	
Clopidogrel Loading Dose[*]		
0 mg (pretreatment)	24.0 (-1.4 – 49.4)	0.27
300 mg	48.3 (9.5 – 87.2)	
600 mg	29.2 (20.2 – 38.1)	
Time to assessment from loading/last dose of clopidogrel[*]		
<12 h	31.1 (20.4 – 41.9)	0.62
12-18 h	28.7 (16.3 – 41.0)	
>18 h	33.7 (-66.3 – 133.7)	
Platelet reactivity cut off for defining HPR (tertiles)[§]		
15 – 49%	47.3 (31.9 – 62.7)	< ***0.01***
50 – 64%	30.7 (20.4 – 41.0)	
65 – 70%	21.6 (12.8 – 30.3)	
Proportion of ACS patients (tertiles)		
0 – 25%	37.1 (20.4 – 53.8)	0.34
25 – 80%	27.9 (5.3 – 50.4)	
80 – 100%	32.0 (20.3 – 43.7)	

Intergroup comparisons were done with the Kruskal-Wallis test. Continuous variables (platelet reactivity cut off and the proportion of ACS patients) were divided into tertiles for the comparison. ACS: acute coronary syndrome. [*]: The POPULAR study[31] was not included as recruited patients received three different clopidogrel regimens. [§]: While LTA_{ADP}, MEA_{ADP} and VASP measures platelet reactivity in a 0 to 100% scale, results of the $VerifyNow_{P2Y12}$ device are scaled from 0–400. Thereby, results of the $VerifyNow_{P2Y12}$ assay were normalized to a 0–100 scale by dividing actual values by 4.

Table 5.1.2 Prevalence of high on-clopidogrel platelet reactivity (HPR).

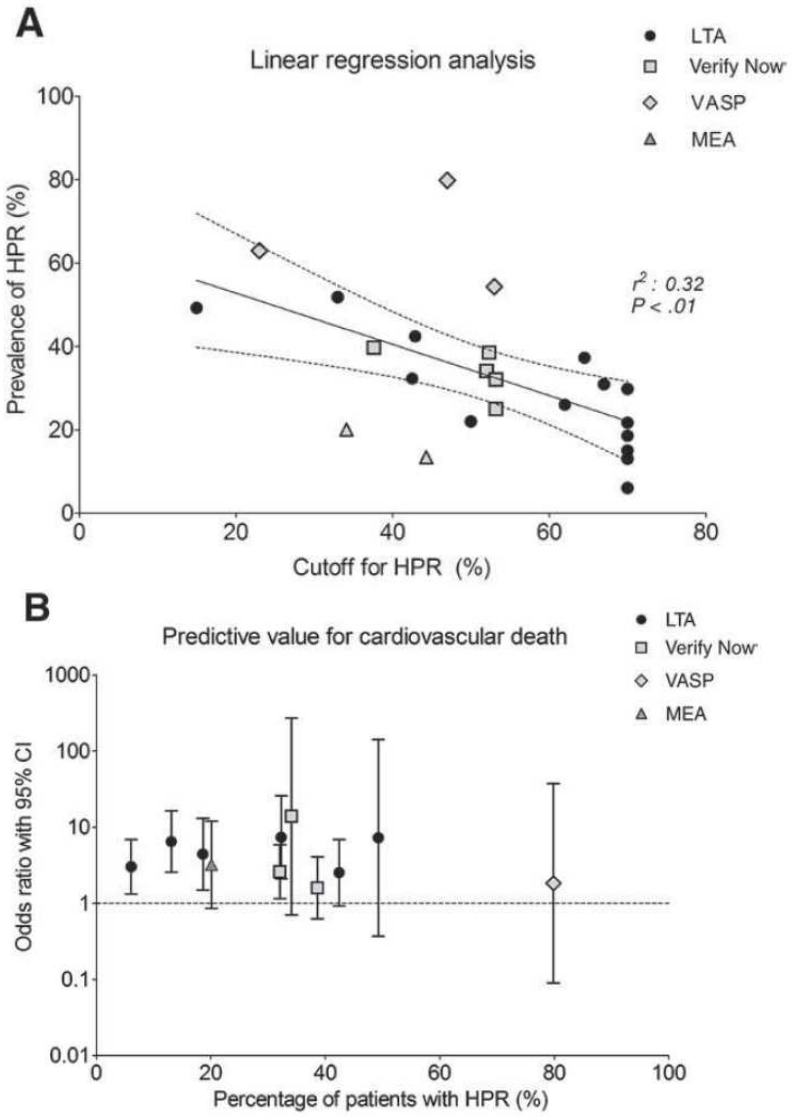
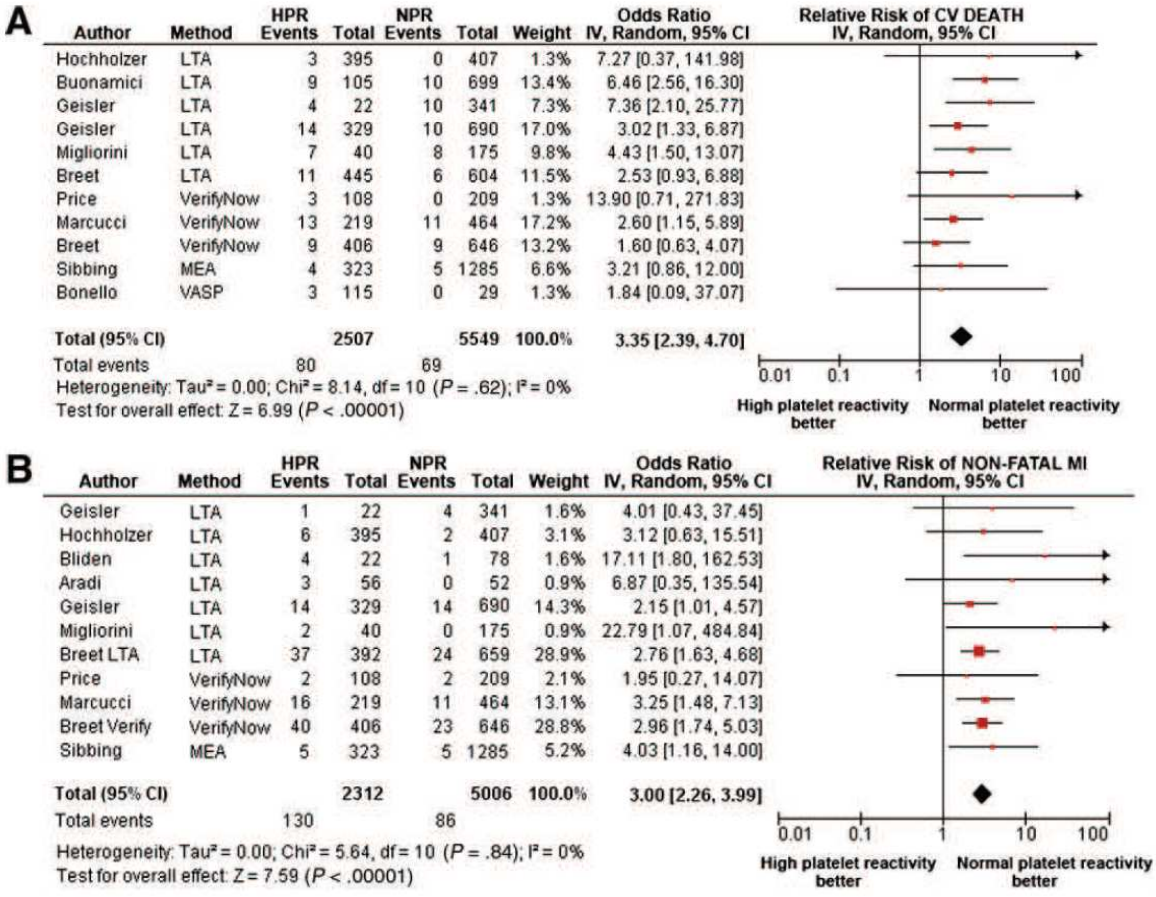


Figure 5.1.1 Impact of the methodological heterogeneity in platelet aggregation tests. A, Linear regression analysis between the selected cut off and the prevalence rate for high platelet reactivity (HPR). Whereas LTA_{ADP} and VASP measure platelet reactivity in a 0% to 100% scale, results might range between 18 and 435 PRU in case of the verifyNow$P2Y_{12}$ and between 0 and 122 U in case of MEA_{ADP} assay according to the description of the manufacturer. Thereby, results of the VerifyNow-$P2Y_{12}$ and MEA_{ADP} assays were normalized to a 0 to 100 scale, where the lowest potential value (18 PRU and 0 U) reflects 0% and the highest potential value (435 PRU and 122 U) means 100%. B, The impact of the prevalence rate of HPR on the relative risk of CV death.

5.1.3 Prognostic significance of HPR

Of the 20 studies, 10 reported data on CV death (39;41;44;54;57-60;62) 10 on nonfatal MI (39;41;49;52;54;57-60;62), and 9 on definite or probable ST. (44;52;54;56;57;59-62) Moreover, there were 15 studies that reported a composite event rate of the recurrent ischemic events (CIE) in compliance with our inclusion criteria. (39-41;47-49;51-55;57;58;62) Based on the pooled results, HPR was associated with a significant 3-fold increase in nonfatal MI (OR 3.00 [2.263.99], P < .00001) (Figure 5.1.2, B), a 4-fold increase in definite/probable ST (OR 4.14 [2.74-6.25], P < .0001) (Figure 5.1.2, C), and a 5-fold increase in the rate of composite ischemic events (OR 4.95 [3.34-7.34], P <.00001) (Figure 5.1.3).



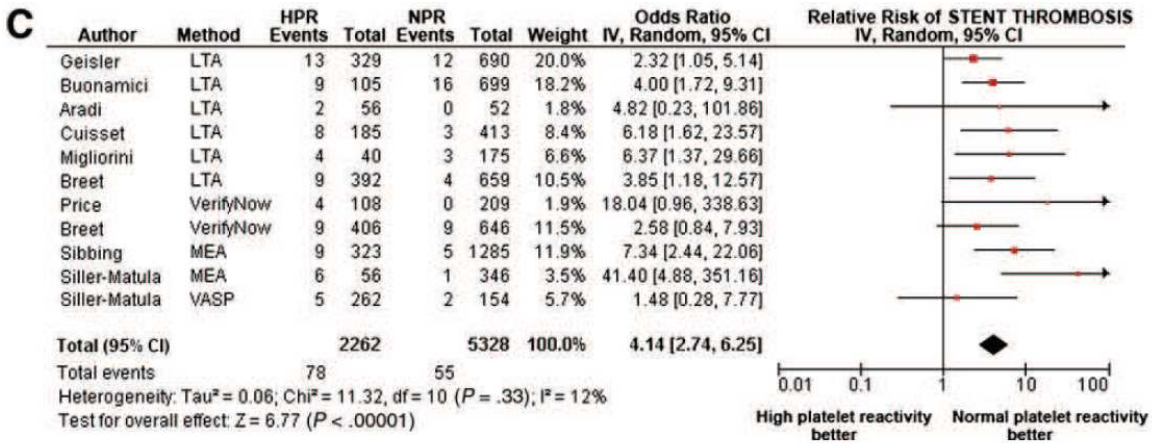


Figure 5.1.2 Impact of HPR on the occurrence of CV death A, nonfatal MI B, and definite or probable ST C. The ORs with 95% CIs were calculated from event frequencies with the random-effect model via generic inverse variance weighting. NPR, Normal platelet reactivity.

Importantly, patients with HPR had a 3.4-fold increase in CV mortality compared with those with normal on-clopidogrel ADP reactivity (OR 3.35 [2.39-4.70], P < .00001) (Figure 5.1.2, A). When the subgroup of studies using receiver operating characteristic (ROC)–defined cut offs for HPR was analyzed separately, similar outputs were gained (CV death 2.34 [1.40-3.92], MI 2.89 [2.07-4.04], ST 4.75 [2.13-10.63], and CIE: 3.06 [2.074.51]; P < .001 in all cases). Although there were large methodical heterogeneity among the platelet function assays as well as in the selected cut offs for HPR, the predicted risk for CV death, nonfatal MI, and ST was not heterogeneous between studies (Figure 5.1.2). On the other hand when the predictive value of each assay was analyzed separately, only LTA-defined HPR was significantly associated with CV death, MI, and ST (death: 4.18 [2.706.46], MI: 2.93 [1.97-4.35], ST: 3.66 [2.32-5.78]; P < .0001 in all cases).

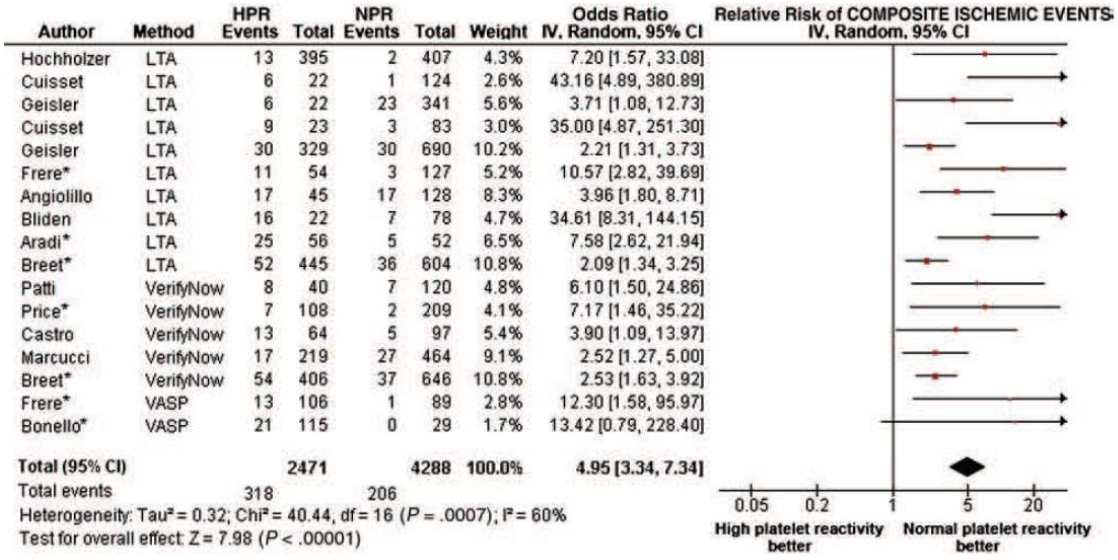


Figure 5.1.3 Impact of HPR on the occurrence of CIE. Where possible, adjusted ORs with 95% CIs were used in the random-effect model via generic inverse variance weighting. *Studies not reporting adjusted ORs.

Because of the large differences in the methodology, terminology, as well as the patient selection and follow-up, we performed subgroup analyses among the studies that reported composite outcome results (Figure 5.1.4). The analysis confirmed that all of the selected ADP-specific assays were able to predict the occurrence of CIE, and the worse prognosis of patients with HPR was consistent regardless of the clinical presentation and the length of follow-up. Notably, there was significant heterogeneity in the results between studies using optical aggregometry; however, the more standardized methods, such as the VerifyNow and VASP assay, showed more homogenous findings.

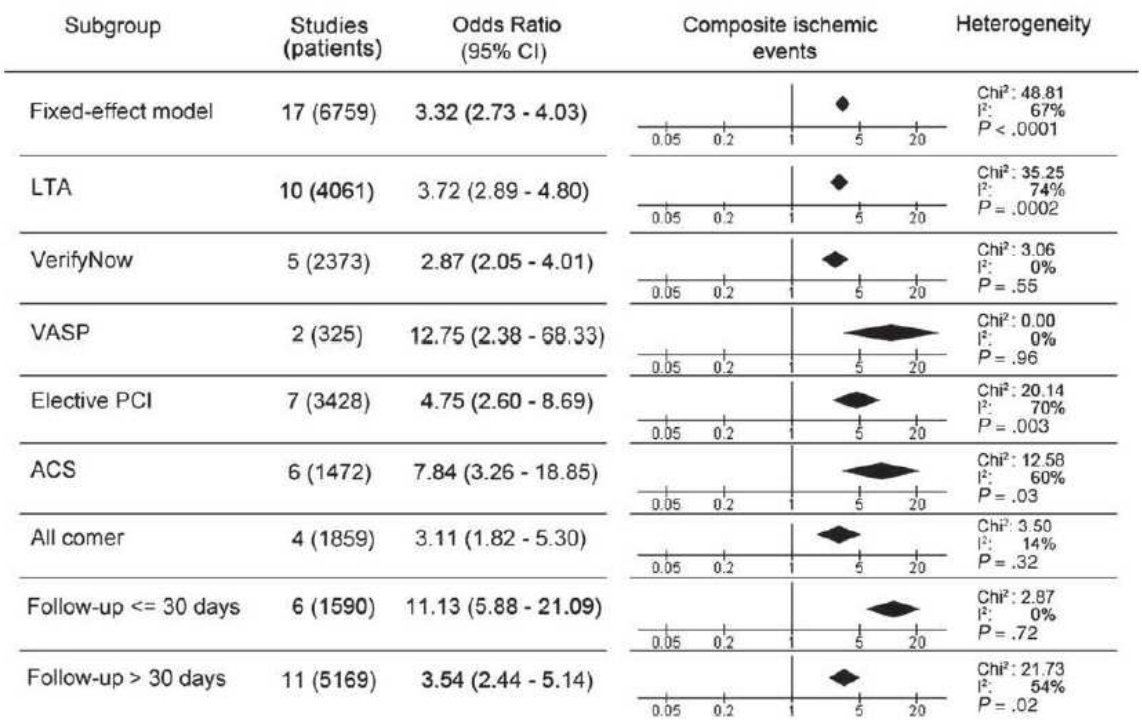


Figure 5.1.4 Sensitivity and subgroup analyses among studies that linked post-treatment platelet reactivity to the occurrence of composite ischemic events.

5.2 Comparison of conventional aggregometry (LTA) with vasodilator stimulated phosphoprotein phosphorylation assay (VASP)

5.2.1 Correlation between LTA and VASP-PRI measurements

One hundred twenty-one patients fulfilled the entry criteria and were enrolled into the study. Baseline clinical characteristics of the recruited patients are depicted in Table 5.2.1. Among these patients, 242 VASP and LTA measurements were performed: the first assessments were done 19±2 hours after receiving a 600-mg loading dose of clopidogrel and maintenance-phase samples were collected at 25±2 days after PCI. After the administration of a 600-mg loading dose of clopidogrel, all platelet function parameters demonstrated high interindividual variability (Agg_{max}: 29.1±14.4; Agg_{late}: 9.4±18.7; disAgg: 71.5±32.4; AUC: 67.6±55.0; VASP-PRI: 48.3±21.3) that also persisted in the maintenance period (Agg_{max}: 29.6±12.7; Agg_{late}: 8.7±16.6; disAgg: 72.2±30.9; AUC: 67.7±49.3; VASP-PRI: 47.9±19.6). Based on the LTA measurements, high correlation was found between the maximal and late aggregation values ($p<0.001$; Spearman's ρ: 0.91). When LTA values were compared to VASP-PRI, significant, moderate-strength correlations were registered without marked difference among the parameters (Agg_{max}: $\rho=0.47$; Agg_{late}: $\rho=0.45$; disAgg: $\rho= -0.44$; AUC: $\rho=0.50$). Notably, the efficacy of aspirin therapy, measured by epinephrine 10 μM, did not correlate to VASP-PRI. ($p=0.75$; $\rho=-0.24$). In the univariate linear regression analyses, all variables of the LTA curve showed similar, significant relationship with VASP assessments. In the multivariate model, AUC proved to be the independent linear predictor of VASP-PRI. To corroborate these results, logistic regression analyses were performed with HPR as a dependent variable. Based on the results, estimates of LTA were similar; however, AUC was selected as the

independent predictor of VASP-defined HPR. When the loading- and the maintenance phase outputs were analyzed separately, similar results were obtained without any difference between the two time points.

Age (years, ± SD)	61.98 ± 8.91
Male gender (*n*, %)	72 (59.5%)
Stable angina (*n*, %)	121 (100%)
Diabetes (*n*, %) INS/OAD/DIET (*n*, %)	38 (31.4%) 13 (10.7%)/16 (13.2%)/9 (7.4%)
Hypertension (*n*, %)	106 (87.6%)
Smoking (*n*, %) Current smoker (*n*, %)	44 (36.4%) 16 (13.2%)
Prior MI (*n*, %)	19 (15.7%)
Prior CABG (*n*, %)	8 (6.6%)
PAD (*n*, %)	6 (5%)
Baseline total cholesterol (mmol/L, ± SD)	4.00 ± 0.95
Baseline serum creatinin (mmol/L, ± SD)	72.56 ± 16.22
Baseline high sensitivity CRP (microm/L, ± SD)	2.28 ± 1.72
Baseline fibrinogen (g/L; ± SD)	3.02 ± 0.82
Baseline leukocyte count (G/L, ± SD)	6.87 ± 1.80
Baseline haemoglobin (g/dl, ± SD)	129.79 ± 12.43
Baseline platelet count (G/L, ± SD)	232.60 ± 65.67

Table 5.2.1 Baseline clinical characteristics of the recruited patients INS, insulin-treated diabetes mellitus; OAD, oral antidiabetic-treated diabetes; MI, myocardial infarction; CABG, coronary artery bypass grafting; PAD, peripheral artery disease.

Bland-Altman plots were used to demonstrate intra-individual agreement among assays in measuring on-clopidogrel platelet reactivity. (Figure 5.2.1) These plots demonstrated that Agg_{late}, disAgg and AUC are underestimating VASP-PRI (bias: -10.6, -19.9 and -15.1, respectively) while platelet reactivity is estimated quite similarly by Agg_{max} (bias: 1.3) and VASP-PRI. The analyses also demonstrated that the underestimation of VASP-PRI by Agg_{late}, disAgg and AUC is largely driven by differences in the low platelet reactivity range, markedly below 50% platelet reactivity. The wide ranges of agreement in case of all LTA variables underscored that there are substantial intra-individual differences between LTA and VASP assessments. (Figure 5.2.1)

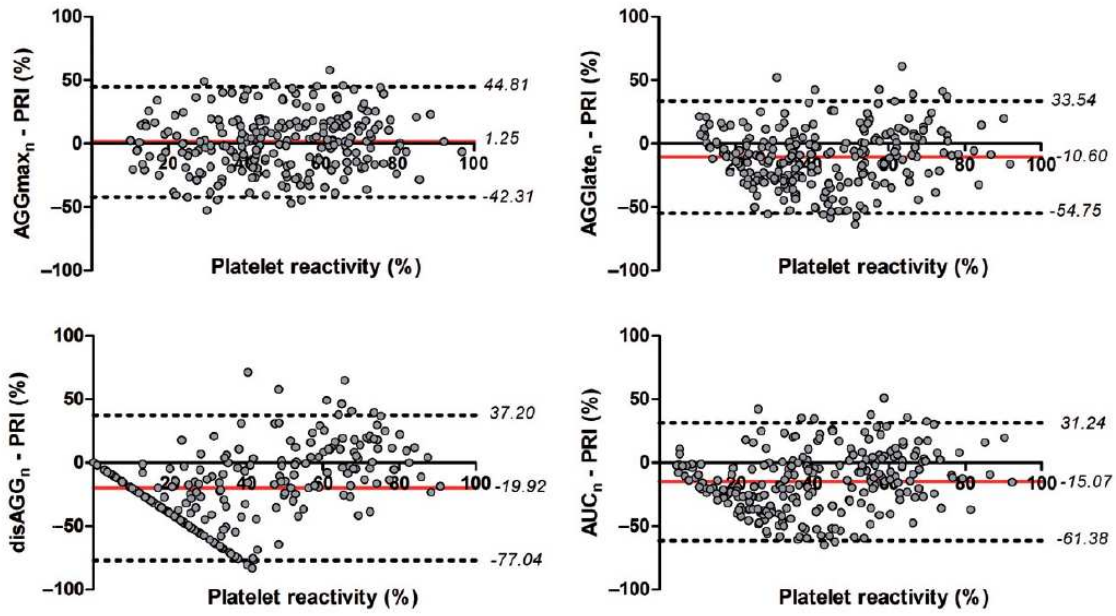


Figure 5.2.1 Bland–Altman plots to demonstrate intra-individual agreement in platelet reactivity. The comparison was performed between vasodilator stimulated phosphoprotein phosphorylation index (VASP-PRI) and estimates of light transmission aggregometry. Bias (red line) is a measure of a systematic error leading to over- or underestimation of a known value (VASP-PRI) by the alternative parameters of the light transmission assessment. Dashed lines represent limits of 95% agreement that form a range within 95% of the measurements can be found. As the principle of Bland-Altman analysis is that both measurements evaluate a parameter on the same scale (platelet reactivity, %), all the light transmission parameters were normalized to the scale of VASP-PRI (from 0% to 100%). Agg_{maxn}, normalized maximal aggregation, Agg_{laten}, normalized 6-minute late aggregation; $disAgg_n$, normalized disAggregation; AUC_n, normalized area under the 6-minute light transmission curve).

5.2.2 Agreement between assays in determining normal and high platelet reactivity

The predictive value of LTA variables in determining HPR as well as the optimal cut off values for the best agreement were evaluated with receiver-operator characteristic (ROC) curve analysis. LTA estimates were equal in predicting HPR with AUC showing the highest area under the ROC curve. Based on the optimal cut off values, we registered significant relationships with moderate-strength agreements between VASP and LTA parameters in classifying patients to normal or HPR categories. (Table 5.2.2) AUC greater than 82% had the highest specificity, yet the lowest sensitivity to identify a patient with a VASP-PRI greater than 50%.

Cut-off	HPR#	Specificity	Sensitivity	Concordant	Discordant	κ
AGGmax >34.5%	39.3%	79.4%	61.3%	71.1%	28.9%	*0.4* '
AGGlate >12%	37.2%	83.2%	62.2%	73.1%	26.9%	*0.45*'
disAGG >63.5%	37.6%	80.2%	63.1%	72.7%	27.3%	*0.44*'
AUC >82 × min	39.7%	86.7%	60.8%	72.3%	27.7%	*0.44*'

Table 5.2.2 Diagnostic accuracy of ROC-defined cut off values in identifying patients as normal (VASP-PRI<50%) or high platelet reactivity (VASP-PRI ≥50%). Optimal cut off values for each aggregometric variable (Agg_{max}, Agg_{late}, disAgg, AUC) were determined with receiver-operating characteristic curve (ROC) analysis according to the highest specificity and sensitivity in the current cohort. *p<0.001, #Notably, 46.6% of the patients had a VASP-PRI ≥50% in the current cohort. HPR, high platelet reactivity; VASP, vasodilator stimulated phosphorylation assay.

5.3 Determining the impact of genetic variants on post-clopidogrel platelet reactivity in patients after elective percutaneous coronary intervention

5.3.1 Genotype distribution

Altogether, genetic samples were available from 189 stable angina patients for analysis. Allelic variants of CYP2C19 and PON-1 were determined in 189 cases, while we had genetic information on ABCB1 genotypes in 181 patients. As we observed significant clinical differences between patients according to the number of CYP2C19 LOF alleles, clinical and procedural characteristics as well as post-procedural laboratory findings are depicted according to this classification in the patient group. (Table 5.3.1) Based on these, patients with wild-type (wt) CYP2C19 alleles had higher hemoglobin levels and lower platelet count.

	No CYP2C19 LOF alleles (n=144)	One CYP2C19 LOF allele (n=41)	Two CYP2C19 LOF alleles (n=4)	P
Clinical risk factors				
Age (years, mean±SD)	61.9±8.2	61.0±9.4	64.3±5.1	0.54
Male gender (n, %)	95 (66.0)	20 (48.8)	1 (25%)	*0.04**
Caucasian race (n, %)	144 (100.0)	41 (100.0)	4 (100.0)	NA
Type II diabetes (n, %)	58 (40.2)	11 (26.8)	2 (50.0)	*0.26*
Hypertension (n, %)	121 (84.0)	36 (87.8)	4 (100.0)	0.59
Dyslipidaemia (n, %)	73 (50.7)	23 (56.1)	2 (50.0)	0.83
Smoker (n, %)	52 (36.1)	13 (29.3)	2(50.0)	0.72
Current smoker (n, %)	19 (13.2)	6 (14.6)	1(25.0)	0.78
BMI (kg/m^2; mean±SD)	29.7±4.8	27.8±4.4	34.1±13.4	0.35
Prior PCI (n, %)	11 (7.6)	3 (7.3)	0 (0.0)	0.85
Prior CABG (n, %)	17 (11.8)	3 (7.3)	0 (0.0)	0.56
Prior stroke (n, %)	4 (2.7)	1 (2.4)	0 (0.0)	0.94

Concomitant medication				
Beta-blockers (n, %)	105 (72.9)	34 (82.9)	4 (100.0)	*0.22*
ACE inhibitors (n, %)	75 (52.1)	18 (43.9)	3 (75.0)	0.40
Angiotensin receptor blockers (n, %)	36 (25)	7 (17.1)	1 (25.0)	0.57
Ca-channel blockers (n, %)	44 (30.5)	12 (29.3)	1 (25.0)	0.96
Statins (n, %)	107 (74.3)	28 (68.3)	4 (100.0)	0.36
Non-CYP metabolized statins (n, %)	11 (7.6)	2 (4.9)	0 (0.0)	0.71
Proton pump inhibitors (n, %)	36 (25.0)	7 (17.1)	1 (25.0)	0.57
150 mg clopidogrel[#]	21 (14.6)	11 (26.8)	1 (25.0)	*0.18*
Procedural characteristics				
Time between clopidogrel LD and LTA assessment (h; mean±SD)	20.3±3.7	21.0±4.0	19.9±2.3	0.81
Radial approach (n, %)	124 (86.1)	35 (85.4)	4 (100.0)	0.72
DES implantation (n, %)	97 (67.4)	30 (73.2)	3 (75.0)	0.75
Total stent length (mm; mean±SD)	32.6±17.3	29.4±14.6	24.8±12.1	0.61
Smallest stent diameter (mm; mean±SD)	3.3±0.4	3.2±0.4	3.6±0.5	*0.14*
Contrast amount (ml; mean±SD)	216.8±90.3	216.6±121.0	205.7±42.5	0.84
Post-procedural laboratory findings				
Fibrinogen (g/l; mean±SD)	3.2±0.8	3.3±0.9	2.9±0.9	0.74
Leukocyte (G/l; mean±SD)	7.0±1.8	6.8±1.8	6.3±2.3	0.59
Hemoglobin (g/l; mean±SD)	133.2±14.2	127.0±14.1	113.8±5.4	*<0.01**
Platelet count (G/l; mean±SD)	221.4±63.0	256.2±81.7	256.0±30.5	*0.01**
Mean platelet volume (fl; mean±SD)	8.8±1.8	8.5±1.3	7.8±2.3	0.50
Serum creatinin (μmol/l; mean±SD)	77.7±53.5	69.8±18.3	76.3±28.5	*0.26*
Total cholesterol (mmol/l; mean±SD)	4.2±1.3	4.3±1.1	4.2±0.7	0.87
High sensitivity CRP (mg/l; mean, SD)	2.9±3.1	2.8±2.9	2.4±3.0	0.93
GOF-carriers (n, %)	69 (47.9)	14 (34.2)	0 (0.0)	*0.06*
CYP2C19 *1/*17 (n, %)	41 (28.5)	0 (0.0)	0 (0.0)	*<0.01**
CYP2C19 *2/*17 or *3*17 (n, %)	0 (0.0)	14 (34.2)	0 (0.0)	*<0.01**
CYP2C19 *17/*17 (n, %)	28 (19.4)	0 (0.0)	0 (0.0)	*<0.01**

Abbreviations: ACE: angiotensin converting enzyme; Agg_{max}: 5-μM ADP-stimulated maximal aggregation value; BMI: body mass index; CABG: coronary artery bypass grafting; CRP: C-reactive protein; DES: drug-eluting stent; DM: diabetes mellitus; GOF: gain-of-function allele; HPR: high on-clopidogrel platelet reactivity; LD: loading dose; LOF: loss-of-function allele; LTA: Light Transmission Aggregometry; MI: myocardial infarction; NPR: normal platelet reactivity; PCI: percutaneous coronary intervention. [*]: Significant difference. [#]: In the original trial, patients with HPR were randomized to receive 150 mg or 75 mg clopidogrel for one month. [13]

Smokers were defined as patients who were smoking in the past 5 years. Current smokers were subjects who were actively smoking at the time of enrollment.

Table 5.3.1 Baseline characteristics of the patient groups.

The observed distributions of different genotypes are presented in Table 5.3.2. Genotype frequencies were similar to previously reported findings in the Caucasian population, and none of the genotype distributions deviated significantly from the Hardy-Weinberg equilibrium.

A. CYP2C19		
Genotypes	Predicted Phenotype	Distribution
*1/*1	Extensive	75 (39.7%)
*1/*17	Rapid	41 (21.7%)
*17/*17	Ultra rapid	28 (14.8%)
*1/*2	Intermediate	27 (14.3%)
*2/*17	Unknown (Poor or Rapid)	13 (6.9%)
*2/*2	Poor	4 (2.1%)
*3/*17	Unknown (Poor or Rapid)	1 (0.5%)
Allele carriage		
Loss-of-function (*2 or *3)		45 (23.8%)
No loss-of-function		144 (76.2%)
Gain-of-function (*17)		83 (43.9%)
No gain-of-function		106 (56.1%)
B. ABCB1 3435C→T		
Genotypes	Predicted Phenotype	Distribution
C/C	High expression	46 (25.4%)
C/T	Intermediate expression	78 (43.1%)
T/T	Low expression	57 (31.5%)
C. ABCB1 2677G→T/A		
Genotypes	Predicted Phenotype	Distribution
G/G	Unknown	51 (28.2%)
A/A	Unknown	12 (6.6%)
T/T	Unknown	35 (19.3%)
G/T	Unknown	81 (44.7%)
T/A	Unknown	2 (1.1%)
D. PON-1 192Q→R		
Genotypes	Predicted Phenotype	Distribution
Q/Q	Low metabolizer	91 (48.1%)
Q/R	Intermediate metabolizer	86 (45.5%)
R/R	Rapid metabolizer	12 (6.4%)

Table 5.3.2 Distribution of genotypes in the patient groups.

5.3.2 CYP2C19 genotypes and platelet reactivity

According to the platelet function results in case of CYP2C19 locus, patients harboring a LOF allele had significantly higher maximal aggregation (32.9±13.6 vs. 26.4±14.5; P=0.01), 6-minute late aggregation (13.7±17.8 vs. 6.3±17.3, p<0.01) and VASP phosphorylation (57.6±20.8 vs. 47.6±.6; P=0.02) than those with wild-type alleles. On the contrary, harboring at least one GOF allele only slightly decreased platelet reactivity (Agg_{max}: 26.4±14.4 vs. 29.2±14.6, P=0.19; Agg_{late}: 6.1±16.8 vs. 9.5±18.3, P=0.19). When patients were divided into groups according to different genotypes, a gene-dose effect appeared. (Figure 5.3.1)

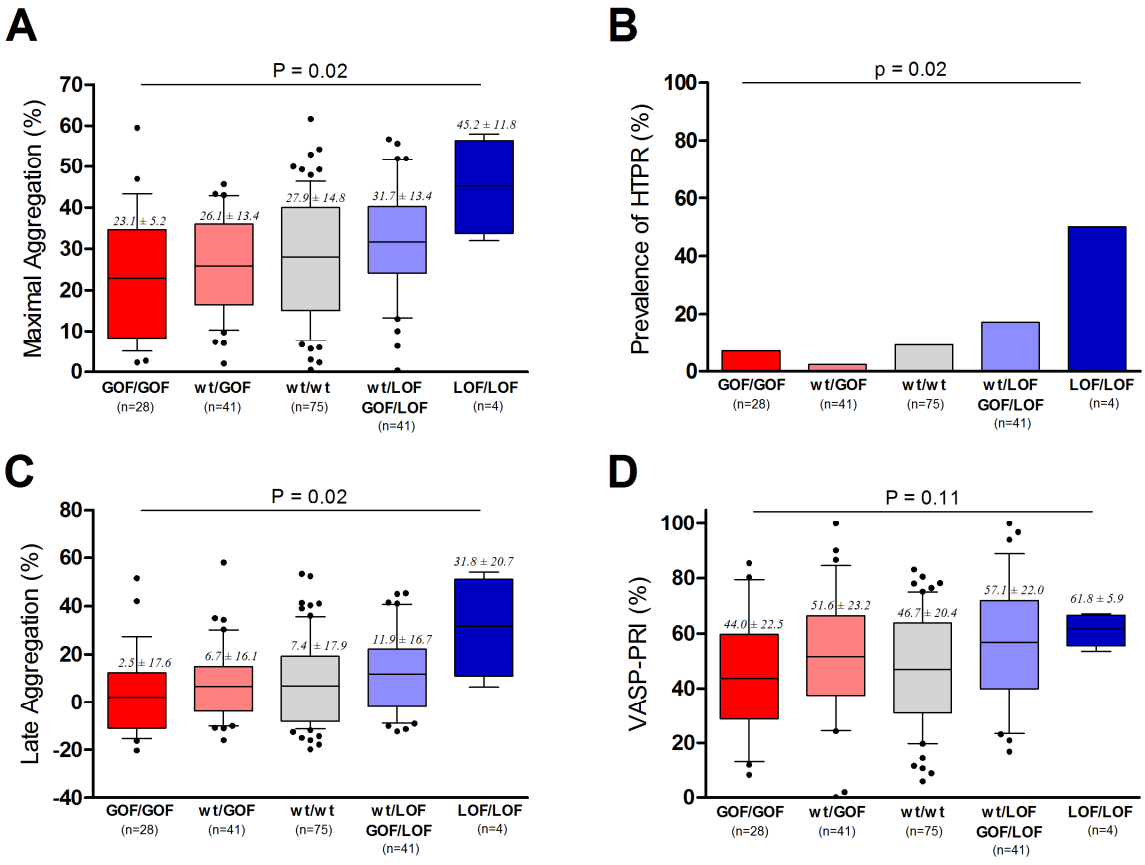


Figure 5.3.1 Comparison in platelet reactivity according to CYP2C19 genotypes.

Post-treatment platelet reactivity was compared with light transmission aggregometry (Panel A and C) and vasodilator stimulated phosphorylation (VASP) assay (Panel D) among patients with different CYP2C19 genotypes. Panel A and C show the maximal (Agg_{max}) and 6-minute late (Agg_{late}) ADP 5 μM-stimulated aggregation values while Panel D depicts VASP-defined platelet reactivity (VASP-PRI). Boxes indicate 25th and 75th percentiles with the mean (line) values, while whiskers denote 10th and 90th percentiles. Mean±SD values are depicted above boxes. Panel B shows the proportion of patients with high post-treatment platelet reactivity (HPR) in each group. GOF: gain-of function allele; LOF: loss-of-function alleles; wt: wild-type allele. The p value shows an inter-group comparison with Kruskall-Wallis test.

Platelet reactivity increased gradually through genotypes according to the following: GOF homozygotes, GOF/wt heterozygotes, wt homozygotes, wt/LOF or LOF/GOF carriers and LOF homozygotes. (Figure 5.3.1 A, C, D) Similarly, the proportion of patients with HPR increased across genotypes. (Figure 5.3.1 B) Despite the increase in platelet reactivity through CYP2C19 genotypes, there was wide variability in platelet function results in all genotype groups. In the linear regression model, carrying a LOF allele explained only 3.6% of the observed variability in Agg_{max} values (R^2: 0.036; $p<0.01$). When both LOF and GOF alleles were included in the model, it explained still only 5.3% of the variability in Agg_{max} values (R^2: 0.053; $p<0.01$). In the binary logistic regression model, patients with a LOF allele had a 3.4-fold odds for HPR; however, the predictive value was only modest based on the c-statistic with a positive predictive value of only 20% and a negative predictive value of 93%. (Table 5.3.3, 5.3.4) On the other hand, harboring a GOF allele was not significantly associated with a

reduced rate of HPR. (Table 5.3.3) The positive and negative predictive values of not having HPR with a GOF allele were 93% and 12%, respectively.

Tested variable	Odds ratio	95% CI	P
A. Univariate binary logistic models:			
CYP2C19 LOF carrier (*1*2 or *2*2)	3.35	1.27 – 8.86	0.02
CYP2C19 GOF carrier (*1*17 or *17*17)	0.56	020 – 1.54	0.26
CYP2C19 LOF homozygote (*2*2)	9.82	1.30 – 74.23	0.03
ABCB1 low expressor (3435 CC)	2.20	1.09 – 4.17	0.26
PON-1 192R carrier (QR or RR)	2.14	0.78 – 5.89	0.14
A. Multivariate binary logistic model:			
CYP2C19 LOF carrier (*1*2 or *2*2)	3.67	1.34 – 9.99	0.01
GOF: gain-of-function, LOF: loss-of-function; PON-1: paraoxonase-1.			

Table 5.3.3 Genetic predictors of high post-clopidogrel platelet reactivity.

Test Result Variables	AUC	95% CI	P
LOF+GOF+ABCB1	0.697	0.558 - 0.837	0.006
LOF+GOF	0.670	0.538 - 0.802	0.018
LOF	0.639	0.495 - 0.783	0.053
ABCB1	0.630	0.484 – 0.776	0.070
PON-1	0.583	0.448 - 0.719	0.247
GOF	0.559	0.422 - 0.696	0.415

GOF: gain-of-function, HPR: high on-treatment platelet reactivity; LOF: loss-of-function; PON-1: paraoxonase-1.

Classification: ***GOF:*** *17-carriers vs. non-carriers; ***LOF*****:** *2 or *3 carriers vs. non-carriers; ***ABCB1:*** 3435 CC carriers vs. CT and TT; ***PON-1*****:** 192 RR and QR vs. QQ carriers; ***LOF+GOF:*** *17*17 or *1*17 vs. *1*1 vs. *1*2 or *1*3 or *2*17 or *2*2; ***LOF+GOF+ABCB1:*** CYP2C19 *17 carriers and 3435 CC non-carriers vs. CYP2C19 *1*1 and 3435 CC non-carriers vs. CYP2C19 *1*1 and 3435 CC carriers vs. CYP2C19 *2 or *3 carriers and 3435 CC non-carriers vs. CYP2C19 *2 or *3 carriers and 3435 CC carriers.

Table 5.3.4 Predictive values of different genotypes on HPR.

5.3.3 PON-1 genotypes and platelet reactivity

Based on the results of LTA and VASP assessments, we found lack of evidence of association between PON-1 192 Q→R polymorphism and post-clopidogrel platelet reactivity. (Figure 5.3.2) The proportion of patients with HPR also did not differ significantly between genotypes, with QQ carriers showing the numerically lowest rates of HPR (QQ: 6.5%; QR: 12.5%; RR: 16.7%; P=0.29). (Figure 5.3.2)

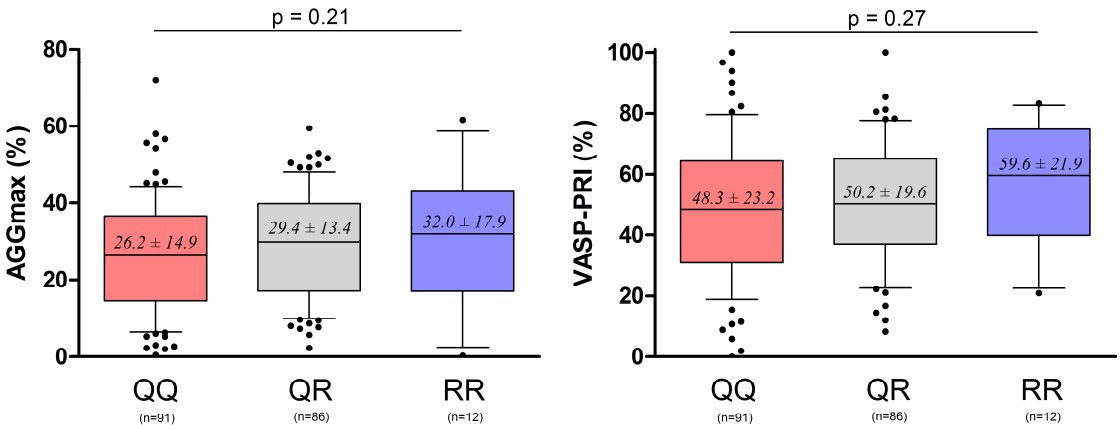


Figure 5.3.2 Comparison in platelet reactivity according to PON-1 genotypes.

Post-treatment platelet reactivity was compared according to different paraoxonase-1 (PON-1) genotypes with light transmission aggregometry (maximal aggregation, Agg_{max}) and vasodilator stimulated phosphorylation (VASP-PRI). Boxes indicate 25th and 75th percentiles with the mean values (line), while whiskers denote 10th and 90th percentiles. Mean±SD values are depicted in boxes. The p value shows an inter-group comparison with Kruskall-Wallis test.

5.3.4 ABCB1 genotypes and platelet reactivity

There were no significant differences in Agg_{max}, Agg_{late} and VASP-PRI values regarding ABCB1 3435 and 2677 genotypes. (Figure 5.3.3)

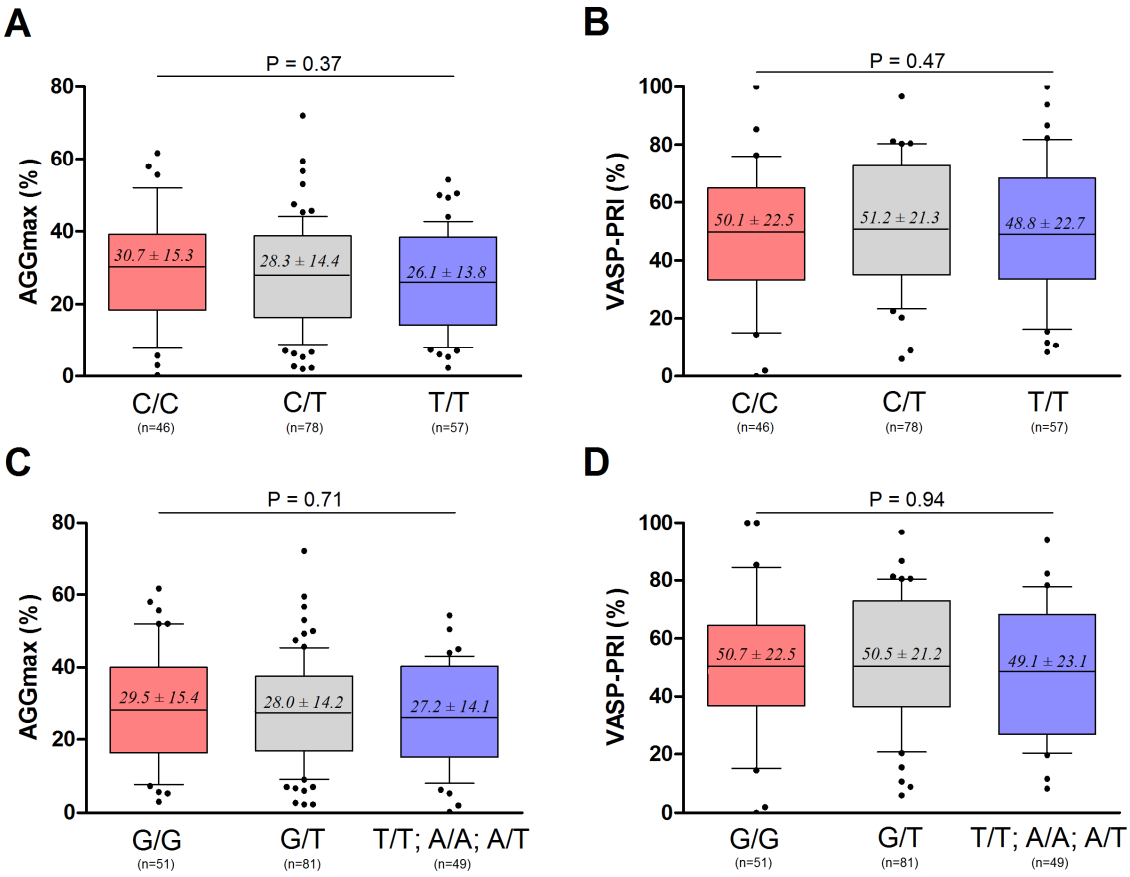


Figure 5.3.3. Comparison in platelet reactivity according to ABCB1 genotypes.

Post-treatment platelet reactivity was compared with light transmission aggregometry (Panel A, C) and vasodilator stimulated phosphorylation (VASP) assay (Panel B, D) in case of 3435C→T (Panel A, B) and 26677G→T/A (Panel C, D) single nucleotide polymorphisms of the ABCB1 gene. Panel A and C show the maximal ADP 5 µM-stimulated aggregation values while Panel B and D depicts VASP-defined platelet reactivity (VASP-PRI). Boxes indicate 25th and 75th percentiles with the mean values (line), while whiskers denote 10th and 90th percentiles. Mean±SD values are depicted in boxes. The p value shows an inter-group comparison with Kruskall-Wallis test.

In fact, low expresser patients with 3435 T/T genotype had numerically lower Agg_{max} values than the high expresser C/C carriers (30.7±15.3 vs. 26.1±13.8; P=0.11). In parallel, 3435 C/C genotypes were associated with a higher rate of HPR (9 [19.6%] vs. 9 [6.7%], P=0.02); but the predictive value of 3435 C/C genotype on HPR was poor. (Table 5.3.3, 5.3.4)

In the multivariate binary logistic regression model, including ABCB1, PON-1 and CYP2C19 genotypes, the carriage of CYP2C19 LOF alleles proved to be the independent determinant of HPR. (Table 5.3.3) This was also confirmed by the ROC analysis, in that CYP2C19 LOF allele carriage had the highest predictive value in forecasting HPR. (Table 5.3.4) Combining the genotype information from LOF and GOF alleles increased the ability to predict HPR; however, the highest area under the curve value was obtained when ABCB1 3435 CC carrier status was added to CYP2C19 genetic information. (Table 5.3.4)

5.3.5 Clinical outcome

During the one year of follow-up, there was one CV death (0.5%), three MIs (1.5%), one probable stent thrombosis (0.5%) and 15 (7.9%) unplanned TVRs. No stroke was recorded during follow-up. Comparing patients with any CYP2C19 LOF alleles to non-LOFcarriers no significant differences in the rate CV death, MI or TVR (Kaplan-Meier estimate: 13.8% vs. 11.1%, HR: 1.24 95%CI: 0.44-3.47, P=0.69) was observed. However, when patients were grouped according to the number of the LOF alleles patients with two LOF alleles had a more than 7-fold unadjusted relative risk to CV death, MI or unplanned TVR (HR: 7.22, 95%CI: 1.61-32.65, P=0.01). When we adjusted for possible confounders between patients, including the presence of a GOF allele, this excess risk remained significant (HR: 9.44, 95%CI: 1.96-45.38, p<0.01). There were no significant differences in the evaluated composite outcome between patients with different ABCB1 3435 and 2677 genotypes (data not shown). Patients with the 3435 CC genotype had numerically higher risk to CV death, MI or TVR compared to

those with C/T or T/T genotype (Kaplan Meier estimate: 14.8% vs. 10.6%, HR: 1.61, 95%CI: 0.60-4.35, P=0.35). Similarly to platelet-function results, there were no differences in clinical outcomes according to PON-1 192 Q→R SNP (data not shown).

6. Discussion

The results of our systematic review and meta-analysis over the prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention, clearly demonstrate, that patients with HPR, detected by an ADP-specific laboratory assay, are at a higher risk for CV death, non-fatal MI, stent thrombosis and recurrent ischemic events. Although there were large differences in the methodology, patient selection and cut off definition between studies, the predicted risk of CV death, MI and ST were not heterogeneous.

Prior to this meta-analysis, numerous observational studies have highlighted that those defined as clopidogrel non/low-responders or with high on-treatment platelet reactivity were at higher risk for recurrent ischemic events. (39-41;44;47-62) However, it is difficult to interpret these results due to the large methodical heterogeneity, the diverse and often arbitrary-defined cut offs for HPR, lack of proven compliance and underpowered sample sizes.

The findings of the current trial confirm results of previous reports. Due to the emerging number and more qualitative meta-analysis studies, big-scale, prospective, adequately powered single-center experiences are available. (57;58;60;62) Therefore, the 3-fold higher risk for non-fatal MI, the 3.4-fold increase in CV death and the 4-fold higher rate for definite / probable ST were demonstrated in almost 9,200 patients. Although some methods such as the MEA_{ADP} and VASP assays were not tested in the study, these results emphasize that the available platelet function tests are not equal in detecting HPR or in predicting clinical outcomes. These inter-assays differences were suggested in prior studies that compared VASP phosphorylation with ADP-stimulated platelet aggregation measurement. According to the

findings, LTA_{ADP} and MEA_{ADP} had better a predictive value than the completely $P2Y_{12}$-specific testing. (51;56;61) Although studies using VASP phosphorylation were under-represented in the current meta-analysis, our results confirmed its poor predictive value regarding CV death and stent thrombosis.

In the meta-analysis, we observed large inter-study and intra-assay heterogeneity in the prevalence of HPR that resulted in a range of 6 to 80%. This was mostly due to the differences in the methodologies and in the diverse definitions of platelet reactivity cut offs. Interestingly, the highest rates of HPR patients were observed in case of the most sensitive $P2Y_{12}$-receptor saturation testing (VASP), whereas whole blood aggregometry (MEA_{ADP}) identified fewer patients with HPR. The higher cut offs in platelet reactivity also resulted in lower prevalence rates of HPR. To evaluate whether the variability in HPR carries clinical consequences, the prevalence of HPR were contrasted to the predictive values regarding cardiovascular mortality. Those studies and devices that found an unexpectedly high prevalence rate of HPR (typically above the mean prevalence rate of 33%) did not predict mortality. It is important to emphasize that on-treatment platelet reactivity follows normal distribution, thereby it is not possible to separate HPR patients on the basis of the laboratory assessment, itself. The cut off for HPR should be assigned on a clinically adjusted basis with receiver-operating characteristic (ROC) curve analysis that might decrease the heterogeneity in the prevalence rate of HPR.

We evaluated the utility and reliability of different parameters of conventional aggregometry and VASP-PRI on monitoring platelet reactivity and predicting clinical outcome, and found that all involved estimates of the LTA assessment are equal in monitoring specific $P2Y_{12}$-receptor inhibition or in predicting VASP-defined high platelet reactivity.

Definition of high platelet reactivity (HPR) as well arbitrary, however it should be defined in consideration of clinical outcome. Based on results of previous study, VASP-PRI greater than 50% was considered as HPR and VASP-PRI lower than 50% was defined as normal platelet reactivity (NPR). (50;51) In the current study we found that, according to the results of ROC analysis, LTA measures were equivalent in predicting HPR.

Following these, by defining the optimal cut off values of LTA parameters normal and HPR patients were separated. When these optimal thresholds were adopted, Agg_{late} showed the highest categorical agreement with VASP-defined NPR and HPR.

However, it is still a matter of debate which is more important: a higher categorical agreement or a better correlation with VASP-PRI. This question should be answered based on clinical evidence. If the association between adverse clinical events and the efficacy of $P2Y_{12}$ 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 cut off 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 study data that may clarify this issue, it remains to be answered.

Our results also affirmed that, the most widely used LTA parameter the Agg_{max}, predicts clinical outcomes after PCI, equally precise as the Agg_{late}, hence superiority of one over the other has not been evidenced. Thereby, the commonly cited hypothesis that Agg_{late} would be more precise compared to other estimates of LTA in monitoring $P2Y_{12}$ receptor inhibition should be rejected. (90)

According to results of the multivariable linear regression analysis, although the differences were minimal between Agg_{late}, Agg_{max}, disAgg and AUC, not Agg_{late}, but AUC proved to be the independent predictor of VASP-PRI. In spite of the significant correlation, there were

considerable intraindividual differences between LTA and VASP assessment. As the VASP assay is completely $P2Y_{12}$-specific, LTA estimates are not precise in expressing $P2Y_{12}$-receptor signalling.

As genetic variations may account for the interidividual differences in the achived anti-platelet efficacy, the impact of CYP2C19, ABCB1 and PON1 genes' allelic variants on Post-Clopidogrel Platelet Reactivity in Patients after PCI were investigated. Results of the study showed that CYP2C19 allelic variants exert a gene-dose effect on post-clopidogrel platelet reactivity: those harboring two gain-of-function alleles (*17) have the lowest average platelet reactivity values, while platelet reactivity increased gradually through ultrarapid - rapid - extensive - intermediate - poor/rapid - poor metabolizer phenotypes reaching the highest degree among carriers of two LOF alleles. (85) Notably, the biggest relative increase in platelet reactivity, hence the highest degree of HPR was seen at the level of patients with two LOF alleles. The role of *2 and *3 alleles were further established in the multivariate analysis, in that CYP2C19 LOF alleles were the independent predictors of HPR. Despite the clear effect of CYP2C19 alleles on platelet function, there was large variability in platelet reactivity according to genotypes and the carriage of a LOF allele explained only 3.6% of this variability.

Although our study was not empowered to demonstrate clinical differences between genotypes, we found that in parallel to platelet function results, patients carrying two LOF alleles had significantly higher risk to ischemic events after elective PCI.

High-risk patients presenting with acute myocardial infarction need potent antiplatelet therapy after PCI and thereby, even a smaller loss in platelet inhibition due to the presence of one LOF allele might manifest in worse clinical outcome. On the contrary, only greater loss in

platelet inhibition - as seen in CYP2C19 *2 and *3 homozygotes - might manifest in worse clinical outcome in low-risk, stable angina patients after PCI.

Common polymorphisms of ABCB1 (3435 C→T; 2677 G→T/A) did not significantly influence platelet function results. Though according to our findings, and in parallel to PLATO genetic substudy, numerically higher risk for HPR and adverse outcome among 3435C/C carriers was observed; but these differences remained non-significant probably due to the small sample size. Although the 2677G→T/A genotype is in linkage disequilibrium with 3435C→T genotype, our findings excluded any interaction of this SNP with clinical outcome in clopidogrel-treated patients. This observation confirms the results of Mega's study. (69)

Due to the results of two independent platelet-function assays, the main allelic variant of PON1 gene (192 Q→R) neither did significantly influence platelet function results, nor associated with clinical outcome.

7. Novel findings of the thesis

Main findings of the thesis can be summarized as the followings:

High on-clopidogrel platelet reactivity (HPR), measured by an ADP-specific platelet function assay is a strong predictor of cardiovascular death, myocardial infarction and stent thrombosis in patients after percutaneous coronary intervention. Although there were large differences in the methodology, patient selection and cut off definition between studies, the predicted risk of cardiovascular death, myocardial infarction and stent thrombosis were not heterogeneous.

The moderate significant correlation with VASP validates LTA for monitoring the efficacy of $P2Y_{12}$ receptor inhibition. LTA parameters were also equivalent in predicting HPR or in classifying patients to VASP-defined categories; however, there might be clinically meaningful differences in the results in certain individuals. Indeed, 6-minute late aggregation (Agg_{late}) is not superior to other estimates of LTA in monitoring the efficacy of $P2Y_{12}$-receptor inhibition.

Genetic variants in CYP2C19 have a gene-dose effect on post-clopidogrel platelet reactivity, with homozygote LOF carriers having the highest risk for HPR and for adverse ischemic events. Neither ABCB1, nor PON-1 genotypes influenced significantly platelet reactivity or outcome.

8. Limitations

In the meta-analysis study, observational trials that are usually unbalanced with regard to baseline clinical characteristics of the patients were included. Although observational studies could better reflect the real-world practice, lack of monitoring drug compliance, underreporting a negative result, and incomplete follow-up make their interpretation more difficult and might carry ascertainment biases. It is also notable that the patients were not treated uniformly regarding the loading doses of clopidogrel and that platelet function assessments were performed at different time points after PCI. One study also enabled the use of different anti-platelet strategies in the maintenance period, whereas none of the selected studies could reliably monitor compliance with the prescribed medication.

The LTA-VASP-PRI comparision study aimed to test pharmacological differences between platelet function estimates; however, it cannot give definite answers regarding the clinical predictive values of the assays. Through several previously published reports comparing LTA with VASP, the fair correlation between the maximal aggregation values and VASP-PRI is not a novel finding. However, our analysis gives a more comprehensive report with the correlation of all possible estimates of LTA (including disaggregation and AUC) with VASP-PRI that has never been evaluated previously.

The main limitation of the genetic study is, that the study was obviously underpowered to determine clear clinical differences between patient groups. The low number of patients carrying two LOF alleles is also a limitation in assessing their platelet reactivity and prognosis. Recent evidence suggests that harboring CYP2C19 GOF alleles might increase the risk of bleeding; however, the sample size of the study did not enable to draw any meaningful

conclusion on bleeding events. Although our results exclude ABCB1 as a major determinant of post-clopidogrel platelet reactivity, we had no pharmacokinetic measurements to directly prove the influence of P-glycoprotein polymorphism on clopidogrel absorption.

9. Summary

Coronary artery disease (CAD) is one of the leading disorder causing aroud 17,5 million death per year and this number is supposed to increase approximately up to 25 million per year by 2020. (91)

However development of stents and the extended use of percutaneous coronary interventions (PCI) have revolutionized the treatment of ischaemic heart disease. In Hungary more than 15000 PCIs are performed in every year.

Giving place of aspirin monotherapy and ticlopidine, dual anti-platelet therapy with aspirin and clopidogrel became the first line recommended treatment in a wide spectrum of patients with ischemic heart disease and in patients after PCI.

As DAPT with aspirin and clopidogrel became widely used large interindividual differences in response to a fix-dose clopidogrel, became evident. (3-5) However due to large methodical heterogeneity and the lack of generally accepted, standardized clinically adjusted bedside tests and well defined cut offs for platelet reactivity values, recommendation for routine screening is still waiting.

In our work we intended to analyze the available heterogenous data and verify the value of platelet functions test in clinical risk assessment, to compare the different estimates of the most widely used optical aggregometry to the most specific vasodilator reactive phosphoprotein assay, as well as to determine the effect of genetic predisposition regarding the efficacy of anti-platelet therapy.

Great hope has been expressed towards the development of personalized medical care strategies in terms of appropriate diagnosis, treatment, and CVD prevention. The issue of validated point-of-care testing and their ability to predict clinical outcomes remains unresolved for antiplatelet drugs. Recent research findings highlight the role of genetic

variation as an important variable for optimizing the response to antiplatelet drugs such as clopidogrel. The goal of personalized medicine is to utilize in part the person's genetic makeup as a guiding information in clinical decision making. In addition, this approach should also include the impact of important non-genetic factors, such as the clinical status of the patient, the environmental factors including diet, and drug–drug interactions. These together with concurrent diseases and clinical presentation defined risk for recurrent ischemic events and for bleeding should optimally be considered in selecting the most appropriate drugs and doses.

10. List of Publications

10.1 Topic related

10.1.1 International articles

Rideg O, Komócsi A, Magyarlaki T, Tőkés-Füzesi M, Miseta A, Kovács LG, Aradi D. Impact of Genetic Variants on Post-Clopidogrel Platelet Reactivity in Patients after Elective Percutaneous Coronary Intervention.

Pharmacogenomics 2011 Sept. E-pub ahead of print IF (2010): 3,876

Rideg O, Háber Á, Botz L, Szücs F, Várnai R, Miseta A, Kovács LG. Pilot study for the characterization of pharmacogenetically relevant *CYP2D6, CYP2C19* and *ABCB1* gene polymorphisms in the Hungarian population. Cell Biochemistry and Function, 2011. in press IF (2010): 1,651

Aradi D, Komocsi A, Vorobcsuk A, Rideg O, Tőkés-Füzesi M, Magyarlaki T, Horváth G I, Serebruany LV. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: Systematic review and meta-analysis.

American Heart Journal, Volume: 160 Issue:3 P: 543-551, 2010. IF: 5,052

Aradi D, Magyarlaki T, Tőkés-Füzesi M, Rideg O, Vorobcsuk A, Sayour A, Horváth IG, Komócsi A. Comparison of conventional aggregometry with VASP for monitoring P2Y12-specific platelet inhibition.

Platelets, Volume: 21 P: 563-570, 2010 IF: 2,117

Aradi D, Rideg O, Vorubcsuk A, Magyarlaki T, Magyari B, Kónyi A, Pintér T, Horvát I, Komócsi A. Investigation 150mg clopidogrel for patients with high on clopidogrel platelet reactivity. European Journal of Clinical Investigation, under review, 2011.

Aradi D, Rideg O, Komócsi A: Platelet function monitoring in patients on clopidogrel. Interventional Medicine & Applied Science Volume: 3(1), P. 32-38, 2011.

Cumulative IF: 12.696

10.1.2 Hungarian abstracts and posters

Aradi D, Rideg O, Magyarlaki T, Tőkés-Füzesi M, Vorobcsuk A, Horváth I, Komócsi A. Genetikai tényezők hatása a clopidogrel kezelt betegek trombocita-reaktivitására és klinikai kimenetélére. Cardiologia Hungarica 41: F71, 2011.

Aradi D, Vorobcsuk A, Sayour A, Magyarlaki T, Tőkés-Füzesi M, Rideg O, Horváth IG, Komócsi A. Optikai Aggregometria mérési eredményeinek összevetése specifikus P2Y12 receptor gátlás áramlási citometriás meghatározásával Cardiologia Hungarica 39: A19, 2009.

Aradi D, Rideg O, Magyarlaki T, Tőkés-Füzesi M, Vorobcsuk A, Horváth I, Komócsi A. Genetikai tényezők hatása a clopidogrel-kezelt betegek trombocita-reaktivitására és klinikai kimenetélére. Cardiologia Hungarica 41: F71, 2011.

Háber Á, Rideg O, Várnai R, Szücs F, Botz L.
Initial Experiences with Paralel CYP2D6/CYP2C19 Geno-Phenotyping in Hungarian Healthy Volunteers. Gyógyszerészet Supplementum 2009/11. Suppl. I., LIII.évf, P-125, pp.: S120.

Háber Á, Szücs F, Rideg O, Osváth P, Fekete S, Botz L. Increased Carrier Prevalence of Deficient CYP2D6 Alleles in Treatment-resistant Depressed Patients. Gyógyszerészet Supplementum 2009/11. Suppl. I., LIII.évf, P-126, pp.: S121.

Rideg O, Aradi D, Magyarlaki T, Tőkés-Füzesi M, Miseta A, Komócsi A, Kovács LG. Impact of Genetic Variants on the Antiplatelet effect of Clopidogrel in Stable Angina Patients after Percutaneous Coronary Intervention, IX MAGE és XVI. Sejt és Fejlődésbiológiai Konferencia, Siófok, 2011.

Rideg O, Aradi D, Magyarlaki T, Tőkés-Füzesi M, Komócsi A, Miseta A, Kovács LG. Impact of Genetic Variants on the Antiplatelet effect of Clopidogrel. Magyar Laboratóriumi Diagnosztikai Társaság 55. Nagygyűlése, Pécs, 2010.

Rideg O, Háber Á, Várnai R, Botz L, Kovács LG. A Magyar populáció CYP2D6 és CYP2C19 allélfrekvenciájának vizsgálata AmpliChip CYP450 teszttel. Magyar Humángenetikai Társaság 2010 évi Konferenciája, Debrecen, 2010.

Rideg O, Háber Á, Várnai R, Botz L, Miseta A, Kovács LG. A Magyar populáció CYP2D6 és CYP2C19 allélfrekvenciájának vizsgálata Amplichip CYP450 teszttel. Magyar Laboratóriumi Diagnosztikai Társaság 55. Nagygyűlése, Pécs, 2010.

10.2 Non Topic related

10.2.1 Articles

Rideg O, Teibert A, Magyarlaki T, Tőkés-Füzesi M, Miseta A, Schmelczer M, Kovács LG. Appearance of multidrug resistance (MDR) as an early predictor of relapse in imatinib treated chronic myeloid leukaemia patients. CLINICA CHIMICA ACTA 355: S285-S285 Suppl. S 2005. IF: 2,149

Rideg O, Csutora P, Magyarlaki T, Teibert A, Nagy T, Kovács LG, Miseta A. Többszörös Gyógyszer-Rezisztencia: Diagnosztikai Megközelítések és Nehézségek. Orvosi Hetilap 146 évf. 20. szám, O: 995-1001, 2005.

Csutora P, Karsai Á, Nagy T, Vas B, Kovács LG, Rideg O, Bogner P, Miseta A. Lithium induces phosphoglucomutase activity in various tissues of rats and in bipolar patients International Journal of Neuropsychopharmacology, Volume: 9 P: 613-619, 2006. IF: 5.889

Szendrei T, Magyarlaki T, Kovács G, Szomor A, Nagy A, Molnár L, Tőkés-Füzesi M, Rideg O, Poto L, Losonczy H. Multidrug-resistance in chronic lymphocytic leukemia Blood Reviews Volume: 21, Suppl 1, S-140, 2007. IF: 5,922

Szendrei T, Magyarlaki T, Rideg O, Tőkés-Füzesi M, Kovács G, Nagy Á, Szomor Á, Molnár L, Dávid M, Pótó L, Pajor L és Losonczy H Multidrog rezisztencia vizsgálatok chronicus lymphoid leukaemiában. Orvosi Hetilap 149 évf. 4. szám, O: 161-167, 2008.

Nagy T, Balassa A, Frank D, Rab A, Rideg O, Kotek G, Magyarlaki T, Bogner P, Kovács LG, Miseta A. O-GlcNAc modification of proteins affects volume regulation in Jurkat cells European Biophys Journal. Volume: 39 (8) P: 1207-17 2010. IF: 2.387

Toth-Kovacs K, Pamer Z, Rideg O, Kovacs A, Fekete S, Biro Z, Kovacs GL: Association of Alzheimer's disease and Age-related macular degeneration in South-Western Hungary, Spektrum Augenheilkd Volume: 2 P: 96-97 DOI 10.1007/s00717-011-0478-2, 2011. IF: 0.120

10.2.2 Abstracts and Posters

Rideg O, Magyarlaki T, Tőkés-Füzesi M, Nagy T, Schmelzer M, Miseta A, Kovács LG. Quantitative analysis of the bcr-abl and the mdr-1 mRNA at Chronic Myeloid Leukaemia patients by LightCycler PCR.

8th International Symphosium on instrumental analysis, Graz, 2005. (Poster presentation)

Rideg O, Tőkés-Füzesi M, Magyarlaki T, Schmelzer M, Miseta A, Kovács LG.

Bcr-Abl és MDR-1 mRNS mennyiségi analízis Valós idejű LightCycler PCR tecnikával krónikus myeloid leukémiás betegekben.

Magyar Hematológiai és Transzfúziológiai Társaság XX. Kongresszusa, Budapest, 2005. (Poster presentation)

Rideg O, Rab A, Jurkuvenaite A, Varga K, Li Y, Clancy J.P. Sorscher E.J. Collawn J.F. Bebok Zs. Cellular Mechanisms Associated with dF508 CFTR Rescue.

The 20th annual North American Cystic Fibrosis Conference, Denver, 2006. (Poster presentation)

Varga K, Jurkuvenaite A, Rideg O, Rowe SM, Clancy J.P., Sorscher E.J, Bebok Zs, Collawn J.F. dF508CFTR surface stability in human airway epithelial cells.
The 20th annual North American Cystic Fibrosis Conference, Denver, 2006. (Poster presentation)

Rideg O, Tőkés-Füzesi M, Magyarlaki T, Miseta A, Kovács LG. Protrombin FII G20210A és Leiden FV G1691A mutációk vizsgálata valós idejű, multiplex PCR technikával.
Magyar Laboratóriumi Diagnosztikai Társaság 54. Nagygyűlése, Debrecen, 2008. (Poster presentation)

Rideg O, Tőkés-Füzesi M, Magyarlaki T, Miseta A, Kovács LG. Janus-kináz 2 gén VAL617PHE mutáció detektálása krónikus myeloproliferatív kórképekben LightCycler PCR technikával. Magyar Humángenetikai Társaság 2008. évi Konferenciája, Pécs, 2008. (Poster presentation)

Rideg O, Lamár I., Miseta A. Az antidepresszív terápia jelene és jövője.
Magyar Orvosi Laboratóriumi Szakdolgozók Egyesülete XI. Nagygyűlése, Pécs, 2009. (Oral presentation)

Frank D, Szalma J, Rideg O, Nagy T, Miseta A, Olasz L Humán papillomavírusok (HPV) szerepe a szájüregi daganatos megbetegedésekben, Magyar Arc-, Állcsont- és Szájsebészeti Társaság XIII. Kongresszusa, Pécs, 2009. (Poster presentation)

Toth-Kovacs K, Pamer Z, Rideg O, Kovacs A, Fekete S, Biro Z, Kovacs LG. Genetical and clinical association between Alzheimer's dementia and age-related macular degeneration. Joint Congress of the European Society of Ophthalmology (SOE) and the American Academy of Ophthalmology (AAO), Geneva, 2011 (Oral presentation)

Cumulative IF: 16,467

Total Cumulative IF : 29,163

11. Acknowledgements

I am very grateful to Prof. Dr. Attila Miseta and Prof. Dr. Gábor L. Kovács for giving me the chance to work at the University of Pécs, Institute of Laboratory Medicine in a very pleasant environment, for helping me in my scientific progress and I do appreciate their exceptional enthusiasm, productive and inspiring suggestions that advanced my scientific thinking and carrier.

I would also gratefully appreciate the support and assistance of my tutor, Dr. András Komócsi, who enabled to progress and tackle difficulties during the whole scientific period.

I would like to express my special thanks to Dr. Dániel Aradi for his help and support.

I am also thankful to Dr. Tamás Magyarlaki, Dr. Margit Tőkés-Füzesi and Sarolta Bácskai, for their great enthusiasm in helping to accomplish my work. They are not only my colleagues, but very good friends of mine.

Finally, I would like to thank to my family and friends for standing beside me every time I really needed them.

12. References

(1) Lordkipanidze M, Pharand C, Schampaert E, Turgeon J, Palisaitis DA, Diodati JG. A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. Eur Heart J 2007 Jul;28(14):1702-8.

(2) Krasopoulos G, Brister SJ, Beattie WS, Buchanan MR. Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis. BMJ 2008 Jan 26;336(7637):195-8.

(3) Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL, Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. J Am Coll Cardiol 2005 Jan 18;45(2):246-51.

(4) Heptinstall S, Glenn JR, May JA, Storey RF, Wilcox RG. Clopidogrel resistance. Catheter Cardiovasc Interv 2004 Nov;63(3):397-8.

(5) Muller I, Besta F, Schulz C, Massberg S, Schonig A, Gawaz M. Prevalence of clopidogrel non-responders among patients with stable angina pectoris scheduled for elective coronary stent placement. Thromb Haemost 2003 Jun;89(5):783-7.

(6) Wiviott SD, Braunwald E, McCabe CH, Horvath I, Keltai M, Herrman JP, et al. Intensive oral antiplatelet therapy for reduction of ischaemic events including stent thrombosis in patients with acute coronary syndromes treated with percutaneous coronary intervention and stenting in the TRITON-TIMI 38 trial: a subanalysis of a randomised trial. Lancet 2008 Apr 19;371(9621):1353-63.

(7) Rinaldi MJ, Kirtane AJ, Piana RN, Caputo RP, Gordon PC, Lopez JJ, et al. Clinical, procedural, and pharmacologic correlates of acute and subacute stent thrombosis: results of a multicenter case-control study with 145 thrombosis events. Am Heart J 2008 Apr;155(4):654-60.

(8) Kastrati A, Mehilli J, von BN, Dibra A, Hausleiter J, Pache J, et al. Sirolimus-eluting stent or paclitaxel-eluting stent vs balloon angioplasty for prevention of recurrences in patients with coronary in-stent restenosis: a randomized controlled trial. JAMA 2005 Jan 12;293(2):165-71.

(9) Schomig A, Dibra A, Windecker S, Mehilli J, Suarez de LJ, Kaiser C, et al. A meta-analysis of 16 randomized trials of sirolimus-eluting stents versus paclitaxel-eluting stents in patients with coronary artery disease. J Am Coll Cardiol 2007 Oct 2;50(14):1373-80.

(10) Vorobcsuk A, Aradi D, Farkasfalvi K, Horvath IG, Komocsi A. Outcomes of patients receiving clopidogrel prior to cardiac surgery. Int J Cardiol 2010 Nov 26.

(11) Aradi D, Rideg O, Komocsi A. Platelet function monitoring in patients on clopidogrel. Interv.Med.& Appl.Sci. 3[1], 32-38. 2011.

(12) Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. Circulation 2002 Jun 25;105(25):2974-80.

(13) Singh M, Gersh BJ, McClelland RL, Ho KK, Willerson JT, Penny WF, et al. Clinical and angiographic predictors of restenosis after percutaneous coronary intervention: insights from the Prevention of Restenosis With Tranilast and Its Outcomes (PRESTO) trial. Circulation 2004 Jun 8;109(22):2727-31.

(14) Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007 Dec 13;357(24):2482-94.

(15) Virmani R, Farb A. Pathology of in-stent restenosis. Curr Opin Lipidol 1999 Dec;10(6):499-506.

(16) Tan KT, Lip GY. The potential role of platelet microparticles in atherosclerosis. Thromb Haemost 2005 Sep;94(3):488-92.

(17) Topol EJ, Mark DB, Lincoff AM, Cohen E, Burton J, Kleiman N, et al. Outcomes at 1 year and economic implications of platelet glycoprotein IIb/IIIa blockade in patients undergoing coronary stenting: results from a multicentre randomised trial. EPISTENT Investigators. Evaluation of Platelet IIb/IIIa Inhibitor for Stenting. Lancet 1999 Dec 11;354(9195):2019-24.

(18) O'Shea JC, Hafley GE, Greenberg S, Hasselblad V, Lorenz TJ, Kitt MM, et al. Platelet glycoprotein IIb/IIIa integrin blockade with eptifibatide in coronary stent intervention: the ESPRIT trial: a randomized controlled trial. JAMA 2001 May 16;285(19):2468-73.

(19) Feit F, Voeltz MD, Attubato MJ, Lincoff AM, Chew DP, Bittl JA, et al. Predictors and impact of major hemorrhage on mortality following percutaneous coronary intervention from the REPLACE-2 Trial. Am J Cardiol 2007 Nov 1;100(9):1364-9.

(20) Newby LK, Califf RM, White HD, Harrington RA, Van de Werf F, Granger CB, et al. The failure of orally administered glycoprotein IIb/IIIa inhibitors to prevent recurrent cardiac events. Am J Med 2002 Jun 1;112(8):647-58.

(21) Smith SC, Jr., Feldman TE, Hirshfeld JW, Jr., Jacobs AK, Kern MJ, King SB, III, et al. ACC/AHA/SCAI 2005 Guideline Update for Percutaneous Coronary Intervention-Summary Article: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/SCAI Writing Committee to Update the 2001 Guidelines for Percutaneous Coronary Intervention). J Am Coll Cardiol 2006 Jan 3;47(1):216-35.

(22) Silber S, Albertsson P, Aviles FF, Camici PG, Colombo A, Hamm C, et al. Guidelines for percutaneous coronary interventions. The Task Force for Percutaneous Coronary Interventions of the European Society of Cardiology. Eur Heart J 2005 Apr;26(8):804-47.

(23) Husted S, van Giezen JJ. Ticagrelor: the first reversibly binding oral P2Y12 receptor antagonist. Cardiovasc Ther 2009;27(4):259-74.

(24) Wallentin L. P2Y(12) inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. Eur Heart J 2009 Aug;30(16):1964-77.

(25) Scrutinio D, Cimminiello C, Marubini E, Pitzalis MV, Di BM, Rizzon P. Ticlopidine versus aspirin after myocardial infarction (STAMI) trial. J Am Coll Cardiol 2001 Apr;37(5):1259-65.

(26) Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, et al. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. J Am Coll Cardiol 2007 Apr 10;49(14):1505-16.

(27) Verstuyft C, Simon T, Kim RB. Personalized medicine and antiplatelet therapy: ready for prime time? Eur Heart J 2009 Aug;30(16):1943-63.

(28) Bouman HJ, Schomig E, Van Werkum JW, Velder J, Hackeng CM, Hirschhauser C, et al. Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 2011 Jan;17(1):110-6.

(29) CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 1996 Nov 16;348(9038):1329-39.

(30) Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001 Aug 16;345(7):494-502.

(31) Steinhubl SR, Berger PB, Mann JT, III, Fry ET, DeLago A, Wilmer C, et al. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA 2002 Nov 20;288(19):2411-20.

(32) Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet 2001 Aug 18;358(9281):527-33.

(33) Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007 Nov 15;357(20):2001-15.

(34) Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2009 Sep 10;361(11):1045-57.

(35) Gurbel PA, Bliden KP, Butler K, Antonino MJ, Wei C, Teng R, et al. Response to ticagrelor in clopidogrel nonresponders and responders and effect of switching therapies: the RESPOND study. Circulation 2010 Mar 16;121(10):1188-99.

(36) Angiolillo DJ, Saucedo JF, Deraad R, Frelinger AL, Gurbel PA, Costigan TM, et al. Increased platelet inhibition after switching from maintenance clopidogrel to prasugrel in patients with acute coronary syndromes: results of the SWAP (SWitching Anti Platelet) study. J Am Coll Cardiol 2010 Sep 21;56(13):1017-23.

(37) Foussas SG, Zairis MN, Patsourakos NG, Makrygiannis SS, Adamopoulou EN, Handanis SM, et al. The impact of oral antiplatelet responsiveness on the long-term prognosis after coronary stenting. Am Heart J 2007 Oct;154(4):676-81.

(38) Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation 2004 Jun 29;109(25):3171-5.

(39) Hochholzer W, Trenk D, Bestehorn HP, Fischer B, Valina CM, Ferenc M, et al. Impact of the degree of peri-interventional platelet inhibition after loading with clopidogrel on early clinical outcome of elective coronary stent placement. J Am Coll Cardiol 2006 Nov 7;48(9):1742-50.

(40) Cuisset T, Frere C, Quilici J, Morange PE, Nait-Saidi L, Mielot C, et al. High post-treatment platelet reactivity is associated with a high incidence of myonecrosis after stenting for non-ST elevation acute coronary syndromes. Thromb Haemost 2007 Feb;97(2):282-7.

(41) Geisler T, Langer H, Wydymus M, Gohring K, Zurn C, Bigalke B, et al. Low response to clopidogrel is associated with cardiovascular outcome after coronary stent implantation. Eur Heart J 2006 Oct;27(20):2420-5.

(42) Gurbel PA, Bliden KP, Samara W, Yoho JA, Hayes K, Fissha MZ, et al. Clopidogrel effect on platelet reactivity in patients with stent thrombosis: results of the CREST Study. J Am Coll Cardiol 2005 Nov 15;46(10):1827-32.

(43) Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Jukema JW, Huisman MV. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. Am Heart J 2007 Aug;154(2):221-31.

(44) Buonamici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Paniccia R, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. J Am Coll Cardiol 2007 Jun 19;49(24):2312-7.

(45) Aradi D, Komocsi A, Vorobcsuk A, Rideg O, Tokes-Fuzesi M, Magyarlaki T, et al. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: Systematic review and meta-analysis. Am Heart J 2010 Sep;160(3):543-51.

(46) Serebruany V, Cherala G, Williams C, Surigin S, Booze C, Kuliczkowski W, et al. Association of platelet responsiveness with clopidogrel metabolism: role of compliance in the assessment of "resistance". Am Heart J 2009 Dec;158(6):925-32.

(47) Cuisset T, Frere C, Quilici J, Morange PE, Nait-Saidi L, Carvajal J, et al. Benefit of a 600-mg loading dose of clopidogrel on platelet reactivity and clinical outcomes in patients with non-ST-segment elevation acute coronary syndrome undergoing coronary stenting. J Am Coll Cardiol 2006 Oct 3;48(7):1339-45.

(48) Angiolillo DJ, Bernardo E, Sabate M, Jimenez-Quevedo P, Costa MA, Palazuelos J, et al. Impact of platelet reactivity on cardiovascular outcomes in patients with type 2

diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2007 Oct 16;50(16):1541-7.

(49) Bliden KP, DiChiara J, Tantry US, Bassi AK, Chaganti SK, Gurbel PA. Increased risk in patients with high platelet aggregation receiving chronic clopidogrel therapy undergoing percutaneous coronary intervention: is the current antiplatelet therapy adequate? J Am Coll Cardiol 2007 Feb 13;49(6):657-66.

(50) Bonello L, Camoin-Jau L, Arques S, Boyer C, Panagides D, Wittenberg O, et al. Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. J Am Coll Cardiol 2008 Apr 8;51(14):1404-11.

(51) Frere C, Cuisset T, Quilici J, Camoin L, Carvajal J, Morange PE, et al. ADP-induced platelet aggregation and platelet reactivity index VASP are good predictive markers for clinical outcomes in non-ST elevation acute coronary syndrome. Thromb Haemost 2007 Oct;98(4):838-43.

(52) Aradi D, Konyi A, Palinkas L, Berki T, Pinter T, Tahin T, et al. Thienopyridine therapy influences late outcome after coronary stent implantation. Angiology 2008 Apr;59(2):172-8.

(53) Patti G, Nusca A, Mangiacapra F, Gatto L, D'Ambrosio A, Di SG. Point-of-care measurement of clopidogrel responsiveness predicts clinical outcome in patients undergoing percutaneous coronary intervention results of the ARMYDA-PRO (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty-Platelet Reactivity Predicts Outcome) study. J Am Coll Cardiol 2008 Sep 30;52(14):1128-33.

(54) Price MJ, Endemann S, Gollapudi RR, Valencia R, Stinis CT, Levisay JP, et al. Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. Eur Heart J 2008 Apr;29(8):992-1000.

(55) de Miguel CA, Cuellas RC, Diego NA, Samaniego LB, Alonso RD, Fernandez VF, et al. Post-treatment platelet reactivity predicts long-term adverse events better than the response to clopidogrel in patients with non-ST-segment elevation acute coronary syndrome. Rev Esp Cardiol 2009 Feb;62(2):126-35.

(56) Cuisset T, Frere C, Quilici J, Gaborit B, Castelli C, Poyet R, et al. Predictive values of post-treatment adenosine diphosphate-induced aggregation and vasodilator-stimulated phosphoprotein index for stent thrombosis after acute coronary syndrome in clopidogrel-treated patients. Am J Cardiol 2009 Oct 15;104(8):1078-82.

(57) Geisler T, Zurn C, Simonenko R, Rapin M, Kraibooj H, Kilias A, et al. Early but not late stent thrombosis is influenced by residual platelet aggregation in patients undergoing coronary interventions. Eur Heart J 2010 Jan;31(1):59-66.

(58) Marcucci R, Gori AM, Paniccia R, Giusti B, Valente S, Giglioli C, et al. Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to

ADP detected by a point-of-care assay: a 12-month follow-up. Circulation 2009 Jan 20;119(2):237-42.

(59) Migliorini A, Valenti R, Marcucci R, Parodi G, Giuliani G, Buonamici P, et al. High residual platelet reactivity after clopidogrel loading and long-term clinical outcome after drug-eluting stenting for unprotected left main coronary disease. Circulation 2009 Dec 1;120(22):2214-21.

(60) Sibbing D, Braun S, Morath T, Mehilli J, Vogt W, Schomig A, et al. Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. J Am Coll Cardiol 2009 Mar 10;53(10):849-56.

(61) Siller-Matula JM, Christ G, Lang IM, Delle-Karth G, Huber K, Jilma B. Multiple electrode aggregometry predicts stent thrombosis better than the vasodilator-stimulated phosphoprotein phosphorylation assay. J Thromb Haemost 2010 Feb;8(2):351-9.

(62) Breet NJ, Van Werkum JW, Bouman HJ, Kelder JC, Ruven HJ, Bal ET, et al. Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA 2010 Feb 24;303(8):754-62.

(63) Kuliczkowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, et al. Interindividual variability in the response to oral antiplatelet drugs: a position paper of the Working Group on antiplatelet drugs resistance appointed by the Section of Cardiovascular Interventions of the Polish Cardiac Society, endorsed by the Working Group on Thrombosis of the European Society of Cardiology. Eur Heart J 2009 Feb;30(4):426-35.

(64) Trenk D, Hochholzer W, Muller B, Stratz C, Valina CM, Schmiebusch P, et al. Antiplatelet response to the 150-mg maintenance dose of clopidogrel in patients with insufficient platelet inhibition after clopidogrel loading for elective coronary stent placement. EuroIntervention 2008 Aug;4(2):214-21.

(65) Taubert D, von BN, Grimberg G, Lazar A, Jung N, Goeser T, et al. Impact of P-glycoprotein on clopidogrel absorption. Clin Pharmacol Ther 2006 Nov;80(5):486-501.

(66) Song P, Lamba JK, Zhang L, Schuetz E, Shukla N, Meibohm B, et al. G2677T and C3435T genotype and haplotype are associated with hepatic ABCB1 (MDR1) expression. J Clin Pharmacol 2006 Mar;46(3):373-9.

(67) Rideg O, Háber Á., Botz L., Szücs F., Várnai R., Miseta A., et al. Pilot study for the characterization of pharmacogenetically relevant CYP2D6, CYP2C19 and ABCB1 gene polymorphisms in the Hungarian Population. impress. 2011.

(68) Kim HJ, Hwang SY, Kim JH, Park HJ, Lee SG, Lee SW, et al. Association between Genetic Polymorphism of Multidrug Resistance 1 Gene and Sasang Constitutions. Evid Based Complement Alternat Med 2009 Sep;6 Suppl 1:73-80.

(69) Mega JL, Close SL, Wiviott SD, Shen L, Walker JR, Simon T, et al. Genetic variants in ABCB1 and CYP2C19 and cardiovascular outcomes after treatment with

clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis. Lancet 2010 Oct 16;376(9749):1312-9.

(70) Pocsai Z, Toth Z, Paragh G, Szeles G, Adany R. Rapid genotyping of paraoxonase 55 and 192 mutations by melting point analysis using real time PCR technology. Clin Chim Acta 2003 Jun;332(1-2):31-6.

(71) Wallentin L, James S, Storey RF, Armstrong M, Barratt BJ, Horrow J, et al. Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with ticagrelor versus clopidogrel for acute coronary syndromes: a genetic substudy of the PLATO trial. Lancet 2010 Oct 16;376(9749):1320-8.

(72) Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, et al. Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 2009 Jan 22;360(4):363-75.

(73) Mega JL, Close SL, Wiviott SD, Shen L, Walker JR, Simon T, et al. Genetic variants in ABCB1 and CYP2C19 and cardiovascular outcomes after treatment with clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis. Lancet 2010 Oct 16;376(9749):1312-9.

(74) Hulot JS, Collet JP, Silvain J, Pena A, Bellemain-Appaix A, Barthelemy O, et al. Cardiovascular risk in clopidogrel-treated patients according to cytochrome P450 2C19*2 loss-of-function allele or proton pump inhibitor coadministration: a systematic meta-analysis. J Am Coll Cardiol 2010 Jul 6;56(2):134-43.

(75) Mega JL, Simon T, Collet JP, Anderson JL, Antman EM, Bliden K, et al. Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. JAMA 2010 Oct 27;304(16):1821-30.

(76) Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB, et al. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 2009 Aug 26;302(8):849-57.

(77) Bozina N, Granic P, Lalic Z, Tramisak I, Lovric M, Stavljenic-Rukavina A. Genetic polymorphisms of cytochromes P450: CYP2C9, CYP2C19, and CYP2D6 in Croatian population. Croat Med J 2003 Aug;44(4):425-8.

(78) Scordo MG, Caputi AP, D'Arrigo C, Fava G, Spina E. Allele and genotype frequencies of CYP2C9, CYP2C19 and CYP2D6 in an Italian population. Pharmacol Res 2004 Aug;50(2):195-200.

(79) Tamminga WJ, Wemer J, Oosterhuis B, de Zeeuw RA, de Leij LF, Jonkman JH. The prevalence of CYP2D6 and CYP2C19 genotypes in a population of healthy Dutch volunteers. Eur J Clin Pharmacol 2001 Dec;57(10):717-22.

(80) Arvanitidis K, Ragia G, Iordanidou M, Kyriaki S, Xanthi A, Tavridou A, et al. Genetic polymorphisms of drug-metabolizing enzymes CYP2D6, CYP2C9, CYP2C19 and CYP3A5 in the Greek population. Fundam Clin Pharmacol 2007 Aug;21(4):419-26.

(81) Hunfeld NG, Mathot RA, Touw DJ, van Schaik RH, Mulder PG, Franck PF, et al. Effect of CYP2C19*2 and *17 mutations on pharmacodynamics and kinetics of proton pump inhibitors in Caucasians. Br J Clin Pharmacol 2008 May;65(5):752-60.

(82) Sibbing D, Koch W, Gebhard D, Schuster T, Braun S, Stegherr J, et al. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation 2010 Feb 2;121(4):512-8.

(83) Brandt JT, Close SL, Iturria SJ, Payne CD, Farid NA, Ernest CS, et al. Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. J Thromb Haemost 2007 Dec;5(12):2429-36.

(84) FDA Drug Safety Communication. Reduced effectiveness of Plavix (clopidogrel) in patients who are poor metabolizers of the drug. 2011. http://www.fda.gov/drugs/drugsafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm203888.htm.

(85) Sibbing D, Gebhard D, Koch W, Braun S, Stegherr J, Morath T, et al. Isolated and interactive impact of common CYP2C19 genetic variants on the antiplatelet effect of chronic clopidogrel therapy. J Thromb Haemost 2010 Aug;8(8):1685-93.

(86) van Himbergen TM, van Tits LJ, Roest M, Stalenhoef AF. The story of PON1: how an organophosphate-hydrolysing enzyme is becoming a player in cardiovascular medicine. Neth J Med 2006 Feb;64(2):34-8.

(87) Lakshmy R, Ahmad D, Abraham RA, Sharma M, Vemparala K, Das S, et al. Paraoxonase gene Q192R & L55M polymorphisms in Indians with acute myocardial infarction & association with oxidized low density lipoprotein. Indian J Med Res 2010 Apr;131:522-9.

(88) van der Stelt CA, Van Werkum JW, Seesing TH, Berg JM, Hackeng CM. To adjust or not to adjust the platelet count in light transmission aggregometry in patients receiving dual aspirin/clopidogrel treatment. Platelets 2007 Nov;18(7):550-3.

(89) Bonello L, Paganelli F, Arpin-Bornet M, Auquier P, Sampol J, Dignat-George F, et al. Vasodilator-stimulated phosphoprotein phosphorylation analysis prior to percutaneous coronary intervention for exclusion of postprocedural major adverse cardiovascular events. J Thromb Haemost 2007 Aug;5(8):1630-6.

(90) Labarthe B, Theroux P, Angioi M, Ghitescu M. Matching the evaluation of the clinical efficacy of clopidogrel to platelet function tests relevant to the biological properties of the drug. J Am Coll Cardiol 2005 Aug 16;46(4):638-45.

(91) Sibbing D, Koch W, Massberg S, Byrne RA, Mehilli J, Schulz S, et al. No association of paraoxonase-1 Q192R genotypes with platelet response to clopidogrel and risk of stent thrombosis after coronary stenting. Eur Heart J 2011 Apr 28.