Monitoring Antiplatelet Therapy in Patients Undergoing Percutaneous Coronary Intervention

Monitoren van plaatjesremmmende medicatie in patiënten die een percutane coronaire interventie ondergaan

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Nulla tenaci invia est via...
Voor papa en mama
Voor Bastiaan

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Introduction

General introduction and outline of the thesis

Platelet function tests for the monitoring of P2Y12 inhibitors

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1 INTRODUCTION

1.1 Antiplatelet therapy

Dual antiplatelet therapy with aspirin and thienopyridines is the cornerstone in the treatment of patients with acute coronary syndrome (ACS) and in those undergoing PCI with stent-implantation.[1,2] However, the magnitude of on-treatment platelet reactivity is not uniform among individuals, due to a multifactorial origin including clinical, pharmacologic and genetic factors.[3,4]

Clopidogrel is a prodrug that requires conversion by hepatic P450 isoenzymes to its active metabolite. Most of the clopidogrel (85%) is hydrolyzed by carboxylase to an inactive carboxylic acid metabolite, whereas the remaining 15% is transformed rapidly into its active metabolite that is able to exert its antiplatelet response by irreversibly inhibiting the binding of adenosinediphosphate (ADP) to the P2Y12 receptor.[5-7] Recently, paraoxonase-1 (PON1) was identified as the crucial enzyme in clopidogrel bioactivation.[8] Consistent findings across multiple investigations support the association between a lower degree of platelet inhibition, *i.e.* a high on-treatment platelet reactivity (HPR), and an increased risk for the occurrence of thrombo-ischemic events (**Table 1**).[9-14,14,15,15-27] Multiple factors have been associated with high on-treatment platelet reactivity, among which genetic polymorphisms of cytochrome P450 and of the P2Y12 receptor as well as and drug-drug interactions. Consequently, the monitoring of the magnitude of platelet reactivity has gained widespread attention.[2]

Table 1: Studies linking high on-treatment platelet reactivity to ADP to the occurrence of thrombotic events

Study (ref)	n	Platelet function assay	Cut-off	Setting	Clinical Endpoint
Matetzky 2004 (8)	60	LTA 5 µmol/L ADP	Aggregation in upper quartile	pPCI in STEMI	6-month Cardiovascular Events
Gurbel 2005 (21)	192	LTA 20 µmol/L ADP	Aggregation in upper quartile	PCI	6-month MACE (death, MI, UAP, stroke)
Gurbel 2005 (22)	120	LTA 5 µmol/L ADP	Magnitude of inhibition	Elective PCI	Peri-procedural myocardial necrosis
Hochholzer 2006 (9)	802	LTA 5 µmol/L ADP	Aggregation > median	Elective PCI	30-day MACE (death, MI, target lesion revascularisation)
Geisler 2006 (10)	379	LTA 20 µmol/L ADP	Aggregation > 70%	PCI	3-month MACE (death, MI, stroke)
Buonamici 2007 (11)	804	LTA 10 µmol/L ADP	Aggregation > 70%	PCI with DES	6-month Definite/probable ST
Frere 2007 (12)	195	LTA 10 µmol/L ADP	Aggregation>70%	PCI in ACS	30-day Recurrent ischemia
Bliden 2007 (23)	100	LTA 5 µmol/L ADP	Aggregation >50%	Elective PCI	1-year MACE (death, MI, ST, stroke, ischemia)
Cuisset 2007 (61)	190	LTA 10 µmol/L ADP	Aggregation>70%	PCI in ACS	Peri-procedural myocardial necrosis

Cuisset 2009 (24)	598	LTA 10 µmol/L ADP	Aggregation>67%	PCI in NSTEMI	30-day Definite/Probable ST
Gurbel 2006 (60)	200	LTA 5 µmol/L ADP	Aggregation >40%	Elective PCI	Peri-procedural myocardial necrosis
Breet 2009 (19)	1049 1051	LTA 5 µmol/L ADP LTA 20 µmol/L ADP	Aggregation>42.9% Aggregation>64.5%	Elective PCI	1-year MACE (death, MI, ST and stroke)
Blindt 2007 (13)	99	VASP	PRI>48%	High risk for ST-PCI	6-month Definite ST
Frere 2007 (12)	195	VASP	PRI>53%	PCI in ACS	30-day Recurrent ischemia
Bonello 2007 (14)	144	VASP	PRI >50%	PCI	6-month MACE (death, stroke, ischemia, revascularization)
Bonello 2007 (25)	162	VASP	PRI >50%	PCI	1-month MACE (death, stroke, revascularization)
Bonello 2009 (26)	429	VASP	PRI >50%	PCI	30-days Definite ST
Siller-Matula 2009 (20)	416	VASP	PRI>42%	PCI	6-month Definite/probable ST
Price 2008 (15)	380	VerifyNow P2Y12 cartridge	>235 PRU	PCI with DES	6-month ST (definite, probable, possible), CV death, nonfatal MI
Patti 2008 (17)	160	VerifyNow P2Y12 cartridge	PRU in upper quartile	PCI	30-day MACE (death, MI, target lesion revascularisation)
Marcucci 2009 (16)	683	VerifyNow P2Y12 cartridge	>240 PRU	PCI in ACS	1-year CV death / nonfatal MI
Valgimigli 2009 (56)	147	VerifyNow P2Y12 cartridge	< 40% Inhibition	PCI	Peri-procedural myocardial necrosis
Breet 2009 (19)	1052	VerifyNow P2Y12 cartridge	>236 PRU	Elective PCI	1-year MACE (death, MI, ST and stroke)
Sibbing 2009 (18)	1608	Multiplate	Upper quintile AU*min>416	PCI with DES	30-day Definite ST
Siller-Matula 2009 (20)	416	Multiplate	AU*min>540	PCI	6-month Definite/probable ST
Matetzky 2004 (8)	60	IMPACT-R	HIghest quartile	pPCI in STEMI	6-month Cardiovascular Events
Breet 2009 (16)	606	Plateletworks	Aggregation>80.5%	Elective PCI	1-year MACE (death, MI, ST and stroke)

ADP=adenosinediphophate, LTA=light transmittance aggregometry, PCI=percutaneous coronary intervention, PPCI=primary (urgent) PCI, STEMI= ST-elevation myocardial infarction, MACE=major adverse cardiovascular events, MI=myocardial infarction, UAP=unstable angina, ST=stent thrombosis, DES=drug-eluting stent, ACS=acute coronary syndrome, NSTEMI=non-ST-elevation myocardial infarction, VASP= vasodilator-stimulated phosphoprotein, PRI= platelet reactivity index, PRU= P2Y12 reaction units, CV=cardiovascular

1.2 Platelet function testing

'Gold standard' light transmittance aggregometry (LTA) has been in use for almost 4 decades and the relationship between high-on treatment platelet reactivity as measured by LTA and the occurrence of thrombo-ischemic events is well established.[9-13,22-24,29] However, LTA is not suitable for routine use in clinical practice because of some major limitations including poor reproducibility, long sample processing time, labour intensiveness and the need for specialized technicians.[28] Therefore, several commercial bedside (or "point-of-care") platelet function tests have been introduced for the rapid evaluation of the individual response to antiplatelet therapy.[30] Point-of-care platelet function tests have the advantage that they are easy to use, require minimal sample handling and the test results are instant available, thereby allowing rapid clinical decision-making. The strengths and drawbacks of the currently available platelet function methods are summarized in **Table 2**. In the present overview, we discuss these assays, including their capability to predict clinical outcome and the role of adjusting antiplatelet therapy based on the test results.

2 CURRENTLY AVAILABLE PLATELET FUNCTION TESTS

2.1 Light transmittance aggregometry (LTA)

2.1.1 Description of the test

Light transmittance aggregometry is based upon the optical detection of platelet aggregation in platelet-rich plasma (PRP). A sample of citrated whole blood is collected and centrifuged to prepare PRP. After removing the platelets in the centrifuged tube, platelet-poor plasma (PPP) is obtained from the remaining specimen after high-speed re-centrifugation. The aggregometer passes a beam of light through the samples. The baseline optical density is set with PPP (100%), whereas the amount of light transmitted trough PRP is defined as 0% aggregation. To induce aggregation, multiple agonists such as adenosine diphosphate (ADP), thrombin-receptor-activating peptide (TRAP), arachidonic acid or collagen can be used. Upon stimulation, the light transmittance increases because aggregates form aggregates that fall out of the solution. The change in light transmittance is a measure for the amount of aggregation. The percentage of aggregation is recorded.

2.1.2 Strengths and drawbacks

Light transmittance aggregometry is widely available and is considered the "gold standard". However, it is poorly standardized and different laboratories often use different protocols with either adjusted or non-adjusted PRP and reporting peak as well as late or final aggregation.[31] Other disadvantages of light transmittance aggregometry are the preparation of platelet-rich plasma, the long processing time and the need for a large sample volume. In addition, it is not a whole blood test and the centrifugation might influence the magnitude of platelet aggregation. Furthermore, LTA is based on fibrinogen-GP IIb/IIIa mediated aggregation and ignores shear stress and platelet adhesion, two aspects that also play a key role in the pathophysiology of thrombus formation.

Table 2: Advantages and drawback of platelet function tests monitoring thienopyridine therapy

	Assay Principle	Aspects of platelet function tested	Advantages	Drawbacks	Accurate detection of clopidogrel	Clopidogrel results correlated with outcome
LTA	Change in light transmittance due to aggregation	Macro-aggregation	Gold standard, Widely available	Labour intensive, large sample volume	+	+
VerifyNow	Agglutination of fibrinogen coated beads	Macro-aggregation	Easy to use, low sample volume, no sample preparation, rapid results, whole blood	Relatively expensive	+	+
Plateletworks	Single platelet disappearance after agonist-stimulation	Micro-aggregation	Easy to use, low sample volume, no sample preparation, rapid results, whole blood	Highly time-dependent	+	+
PFA-100	Cessation of blood flow by platelet plug at high shear	Adhesion and aggregation	Easy to use, low sample volume, no sample preparation, rapid results, whole blood	Highly dependent on von Willebrand Factor and hematocrit levels, limited range	-	-
IMPACT-R	Magnitude of shear-induced platelet adhesion	Adhesion and aggregation	Low sample volume, no sample preparation, rapid results, whole blood	Extensive sample handling, no correlation with active metabolite of clopidogrel	+	-
Multiplate	Change in impedance due to aggregation	Macro-aggregation	Easy to use, low sample volume, no sample preparation, rapid results, whole blood	Extensive sample handling, need for trained technician	+	+
VASP	VASP-phosphorylation state	Activation-dependent signaling	Biochemical gold standard for monitoring clopidogrel	Extensive sample handling	+	+
Placor PRT	Cessation of blood flow by platelet plug	Adhesion and aggregation	Easy to use, no sample preparation, rapid results, fully automated, whole blood	Ability to predict clinical outcome needs to be established	Unknown	Unknown
T-Guide	Change in light scattering due to aggegration	Macro-aggregation	Easy to use, no sample preparation, rapid results, fully automated, whole blood	Ability to predict clinical outcome needs to be established	Unknown	Unknown

LTA=light transmittance aggregometry, PFA=platelet function analyzer, VASP= vasodilator-stimulated phosphoprotein

2.1.3 Clinical Utility

A recent study demonstrated that the biological activity of clopidogrel as assessed by LTA provides a good representation of the plasma levels of the active metabolite of clopidogrel and is therefore suitable to monitor the individual response to clopidogrel.[32] Recently, multiple studies have evaluated the clinical relevance of high on-treatment platelet reactivity as measured by LTA.[9-13]

In a prospective study, Hochholzer and colleagues included 802 patients undergoing elective coronary stent implantation. Platelet aggregation was measured by LTA, using ADP in a final concentration of 5 µmol/L as the agonist.[10] Patients were divided into quartiles according to the magnitude of platelet reactivity. The primary endpoint was defined as a composite of death, myocardial infarction (MI) and target lesion revascularization at 30-day follow-up. The occurrence of the primary endpoint significantly differed between the quartiles of platelet reactivity (0.5% in the first vs. 3.5% in the fourth, p=0.034). Furthermore, patients above the median level of platelet reactivity experienced a 6.7 fold higher risk of reaching the combined endpoint (95% CI: 1.52-29.41, p =0.003) and platelet reactivity (per 10% increase) was an independent predictor of 30-day follow-up ($OR_{adjusted}$=1.32; 95% CI: 1.04-1.61, p=0.026).

Geisler and coworkers assessed platelet reactivity using LTA (20 µmol/L ADP) in 379 patients with symptomatic coronary artery disease (CAD) undergoing PCI.[11] Platelet aggregation>70% was defined as high on-treatment platelet reactivity. At 3-month follow-up patients exhibiting high on-treatment platelet reactivity had a significantly higher risk of major cardiovascular (CV) events (CV death, MI and stroke). High on-treatment platelet reactivity was independently associated with the occurrence of major CV events (HR=3.71; 95%CI:1.08-12.69, p=0.037).

Buonamici et al. performed LTA (10 µmol/L ADP) in 804 patients undergoing drug eluting stent implantation.[12] Patients with platelet reactivity above 70% were considered to have high on-treatment platelet reactivity. The primary endpoint was defined as the occurrence of definite or probable stent thrombosis (ST) at 6-month follow-up. The incidence of ST differed significantly between patients with and without high on-treatment platelet reactivity (8.6% vs. 2.3% , p< 0.001) and high on-treatment platelet reactivity was identified as a strong independent predictor of ST (HR=3.08; 95% CI: 1.32 to 7.16, p=0.009).

Most recently the *The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel pretreated patients undergoing elective **PC**I* (the POPular-study) demonstrated in a large cohort of patients (1069) that high on-treatment platelet reactivity as measured with LTA (5 and 20 µmol/L ADP) is able to discriminate between patients with and without CV events at one year follow-up. The primary endpoint (a composite of death, MI, ST and stroke) occurred more frequently in patients with high on-treatment platelet reactivity (11.7% vs 6.0%, p=0.0009 using 5 µmol/L ADP [n=1049] and 12.0% vs 6.2%, p=0.001 using 20 µmol/L ADP [n=1051]), using a Receiver-Operator Characteristics curve derived cut-off level for defining high on-treatment platelet reactivity of 42.9% and 64.5% peak aggregation for 5 and 20 µmol/L ADP, respectively. [20]

2.2 VerifyNow®

2.2.1 Description of the test

The VerifyNow® system (Accumetrics, San Diego, USA) is a whole blood cartridge-based method to determine the magnitude of agonist-induced platelet inhibition.[33,34]

After a citrated tube of whole blood is inserted into the VerifyNow P2Y12 assay, platelets become activated using 20 µmol/L ADP to induce platelet activation and 22 nmol/L prostaglandin E_1 (PGE_1) to inhibit the contribution of P2Y1 receptor stimulation by ADP. As a result, the activated platelets bind via GP IIb/IIIa receptors to fibrinogen-coated beads and cause agglutination. A baseline value of platelet reactivity is provided by a second chamber containing TRAP as a measure of maximal intrinsic platelet activation. Infrared-light transmittance through the chamber increases as the platelet-bead complexes fall out of the solution. The results are reported in P2Y12 reaction units, in a BASE value and in a percentage inhibition.

2.2.2 Strengths and drawbacks

The VerifyNow is a fully automated "true" point-of-care device. It uses a small amount of whole blood and results are obtained within minutes. Nonetheless, platelet function is not assessed under high shear conditions and the VerifyNow single-use P2Y12 cartridges are relatively expensive.

2.2.3 Clinical Utility

A recent study demonstrated that the results of the VerifyNow P2Y12 assay provide a good representation of the plasma levels of the active metabolite of clopidogrel and is therefore suitable to monitor the effect of clopidogrel therapy.[32] Multiple prospective observational studies have established the association between the results of the VerifyNow P2Y12-assay and clinical outcome in patients undergoing PCI.[17,18,20,35]

Price and colleagues were the first to demonstrate a relation between HPR as measured with the VerifyNow P2Y12-assay and the occurrence of post-discharge events after PCI with drug eluting stent (DES) implantation.[36] On-treatment platelet reactivity was measured using the VerifyNow P2Y12 assay in 380 patients. Receiver operator characteristic (ROC) curve analysis was performed to determine the optimal cut-off value in predicting 6-month cardiovascular (CV) death, non-fatal myocardial infarction (MI) or stent thrombosis (ST). Patients above the optimal cut-off (PRU >235) were at significantly higher risk of CV death (2.8 vs. 0%, p=0.04), ST (4.6% vs. 0%, p =0.004) and the composite endpoint (6.5 vs. 1.0%, p=0.008). High on-treatment platelet reactivity was independently associated with the occurrence of the primary endpoint (OR=7.9; 95%CI: 1.60-38.80, p=0.01).

The ARMYDA-PRO study indicated that HPR according to the VerifyNow P2Y12 assay was able to predict ischemia at 30-days.[18] This study, including 160 patients undergoing DES-implantation, demonstrated that patients in the highest quartile according to PRU had a six-fold higher risk of major adverse cardiac events (MACE) at 30-days follow-up (OR=6.1; 95%-CI: 1.1-18.3, p=0.033) as compared to the lowest quartile. In the ARMYDA-PRO, the optimal cut-off level based on ROC-

analysis was PRU>240.

Marcucci et al. observed that the VerifyNow P2Y12 was able to discriminate between patients with and without a primary endpoint (a composite of CV death and non-fatal MI) at one-year follow-up.[17] Six hundred eighty three patients presenting with acute coronary syndrome (ACS) undergoing PCI were tested using the VerifyNow P2Y12 assay. ROC-curve analysis revealed an optimal cut-off value of PRU>240. At one-year follow-up, the primary endpoint occurred more frequently in patients with HPR (HR=2.52; 95% CI: 1.30-5.13, p=0.011). Furthermore, high on-treatment platelet reactivity was independently associated with the occurrence of cardiovascular death (HR=2.55; 95% CI: 1.08-6.07, p=0.034) and nonfatal MI (HR=3.36; 95% CI: 1.49-7.58, p=0.004).

Recently, the POPular-study demonstrated that high on-treatment platelet reactivity as assessed by the VerifyNow P2Y12-assay is able to discriminate between patients with and without CV event at one-year follow-up.[20] ROC-curve analysis revealed an optimal cut-off value of PRU>236. At one-year follow-up, the primary endpoint occurred more frequently in patients with HPR (13.3% vs. 5.7%, p<0.0001 [n=1052]).

2.3 Multiplate®

2.3.1 Description of the test

The Multiplate® (Dynabyte) is based on whole blood multiple electrode aggregometry (MEA) and detects changes in the electrical impedance due to the adhesion and aggregation of platelets on two independent sensor units in the test cuvette.[19,37] A dilution of hirudin anti-coagulated whole blood (1:2 with 0.9% NaCl solution) is stirred for three minutes in the test cuvettes at 37°C. Two different agonist panels are available to monitor the effects of P2Y12 antagonists: the ADP-test (using ADP as agonist) and the high-sensitivity ADP-test (HS ADP-test; using ADP and PGE_1). PGE_1 potentiates the ability of P2Y12 antagonists to inhibit ADP-induced platelet aggregation. After the addition of ADP (6.4 µmol/l) or ADP & PGE_1 (6.4 µmol/l /9.4 nmol/l), the change in impedance owing to the aggregation of platelets is continuously monitored for five minutes. The increase in impedance is transformed to arbitrary aggregation units (AU) that are plotted against time (AU*min). Eight AU correspond with approximately 1 Ohm. MEA test results are reported as either AU and as area under the curve of arbitrary units (AU*min).

2.3.2 Strengths and drawbacks

Multiplate® is widely used in Europe. Only small amounts of whole blood are required and results are available within a couple of minutes. However, platelet function is not assessed under high shear conditions and this test requires pipetting.

2.3.3 Clinical Utility

Sibbing and colleagues carried out a prospective evaluation of MEA in order to assess whether high on-treatment platelet reactivity as measured with MEA is associated with an increased risk

for early ST.[19] Among 1608 consecutive patients undergoing DES implantation, platelet reactivity was assessed using the MEA (ADP-test) and patients were divided into quintiles according to the magnitude of platelet reactivity. The upper quintile (AU>416) was defined as high on-treatment platelet reactivity. Patients in the highest quintile had a nine-fold higher risk of definite ST within 30-days (OR=9.4; 95%CI: 3.1-28.4, p<0.0001), a three-fold higher risk of death (OR=3.2; 95%CI: 0.9-11.1, p=0.07) and a five-fold higher risk of the composite of death or ST (OR=5.1; 95%CI: 2.2-11.6, p<0.001).

Very recently, Siller-Matula and co-workers used the MEA (HS ADP-test) in 416 patients undergoing PCI and recorded the incidence of ST during a 6-month follow-up.[21] ROC-curve analysis demonstrated that MEA was able to identify patients at higher risk for (definite and probable) ST, using a cut-off value of 54 U (540 AU*min). Patients exhibiting high on-treatment platelet reactivity had a 7.4-fold higher risk for ST.

2.4 Plateletworks®

2.4.1 Description of the test

The Plateletsworks® assay (Helena Laboratories, Beaumont, Texas) is based on single platelet disappearance expressed as the platelet count ratio before and after exposure to ADP, to calculate the percentage of platelet inhibition.[38] Whole blood samples are collected in tubes containing K_3-EDTA and tubes containing PPACK with 20 µmol/L ADP. A routine platelet count is performed on each sample. The platelet count in the K_3-EDTA tube is used as reference. As the aggregated platelets exceed the threshold limitations for platelet size (<30fL) after stimulation with ADP, they are no longer counted as individual platelets. The ratio between the aggregated platelets in the agonist sample and the platelet count in the reference tube (ADP/EDTA) x 100% is used as the degree of platelet aggregation.

2.4.2. Strengths and drawbacks

Plateletworks® requires minimal sample preparation, uses a small volume of whole blood and the results are available within minutes. However, rapid performance (within 10 minutes after blood withdrawal) of this assay is required since platelet aggregates formed upon ADP stimulation disaggregate after this time-point resulting in an unreliable test result.[38] Therefore, the use of the Plateletworks® in routine clinical practice might be limited. A possible solution might be placing a cell counter in the catheterization laboratory for rapid measurements between 5 and 10 minutes after blood collection.

2.4.3. Clinical Utility

In the The Ongoing Tirofiban in Myocardial Evaluation (On-TIME) trial, Smit et al used the Plateletworks assay in 463 patients presenting with a STEMI undergoing PCI. Patients undergoing primary PCI were either treated with Tirofiban or placebo. No relation between the magnitude of

platelet reactivity as assessed by the Plateletworks and clinical outcome was found in the On-TIME.[39]

The POPular study is the largest study to date (n=606) that demonstrated a relation between the Plateletworks® ADP assay and clinical outcome. ROC-analysis based on the primary endpoint (a composite of death, MI, ST and stroke) at one-year follow-up revealed an optimal cut-off value of 80.5% aggregation. At one-year follow-up, the primary endpoint occurred more frequently in patients above this cut-off (12.6% vs 6.1%, p=0.005).[20]

2.5 PFA-100 system

2.5.1. Description of the test

The PFA-100® System (Siemens Healthcare Diagnostics Products GmbH, Germany),
measures platelet function, in particular adhesion and aggregation, in whole blood under high shear conditions (5000s^{-1}).[40] The time needed to form a platelet plug occluding the aperture cut into a membrane coated with an agonist is determined and reported as closure time (CT) in seconds, which is inversely related to platelet reactivity.

Various types of cartridges are available. The membrane of the classic cartridge is coated with collagen and either ADP (COL/ADP) or epinephrine (COL/EPI) as agonists and recently a novel PFA-100® test cartridge has been introduced, the final prototype of INNOVANCE® PFA P2Y* This novel test cartridge intents to measure the effect of clopidogrel on platelet function.[41]

2.5.2. Strengths and drawbacks

The PFA-100 is a simple and semi-automated assay that uses whole blood. It mimics the in vivo process of thrombus formation by inducing shear stress. However, several studies have demonstrated that the COL/ADP cartridge is relatively insensitive to the effect of thienopyridine treatment.[42] This might be attributed to the relatively high local concentration of collagen (6µg/ml) and ADP (0.5mmol/L) in the cartridge, under which circumstances clopidogrel might be unable to inhibit the formation of the platelet plug. Furthermore, since the assay is highly affected by the levels of von Willebrand Factor (vWF) and hematocrit, high vWF levels might mask the inhibitory effects of clopidogrel because the shear stress will lead to instant binding of vWF to GPIIb/IIIa.[43]

2.5.3. Clinical Utility

Clinical evaluation of the PFA-100 COL/ADP in monitoring clopidogrel therapy is limited. Thus far, only the POPular-study described performance data of the cartridge in a large cohort of patients undergoing elective PCI.[20] The PFA-100 COL/ADP cartridge (n=812) was unable to discriminate between patients with and without ischemic events at one-year follow-up.
INNOVANCE® PFA P2Y* has not been published yet.

2.6 VASP

2.6.1 Description of the test

The vasodilator-stimulated phosphoprotein (VASP) is an intraplatelet actin regulatory protein. Activation of the platelet P2Y12 receptor by ADP suppresses the production of the intracellular platelet inhibitor cyclic adenosinemonophosphate (cAMP), thereby causing the dephosphorylation of VASP and the activation of glycoprotein IIb/IIIa-receptors (GPIIb/IIIa). Conversely, inhibition of the P2Y12 receptor (by thienopyridines) induces phosphorylation of VASP. VASP-phosphorylation state (VASP-P) thus reflects the magnitude of P2Y12 inhibition.

VASP-P is measured using a standardized flow cytometric assay (commercial assay, BioCytex, Marseille, France), adapted from Schwarz et al.[44] A citrated blood sample is incubated with either PGE1 or with PGE1 and ADP for 10 minutes. PGE1 leads to maximal VASP-phosphorylation, and in a sample with PGE1 and ADP the ability of ADP to counteract the influence of PGE1 on VASP-phosphorylation state is being tested.

The sample is fixed with paraformaldehyde, after which the platelets are permeabilized with non-ionic detergent (Triton X-100). Then, blood samples are incubated with CD61 (platelet identification) and antibodies against VASP-P (FITC). A platelet reactivity index (PRI) is calculated using corrected mean fluorescence intensities (MFIc) in the presence of PGE_1 alone or PGE_1 and ADP simultaneously according to the following equation: $PRI = (MFIc_{PGE1} - MFIc_{PGE1+ADP}) / MFIc_{PGE1}$. High on-treatment platelet reactivity is defined as a high PRI, whereas a low PRI indicates an adequate P2Y12 inhibition.

The PLT VASP/P2Y12 -assay shows a good correlation with light transmittance aggregometry (r=0.72) and it has been demonstrated that VASP has high sensitivity and specificity for clopidogrel treatment.[45]

2.6.2 Strengths and drawbacks

The VASP assay is dependent on the target of thienopyridines (P2Y12), and is therefore considered the "biochemical" gold standard for assessing the effectiveness of P2Y12-receptor blockade.[45,46] It involves low volume samples of whole blood and can be used in the presence of GP IIb/IIIa-therapy. Moreover, in contrast to the other platelet function tests that need to be performed within 2 hours after blood withdrawal, the PRI measured flow cytometric analysis of VASP-P is temporal stable and the sample can be stored at room temperature for 24 hours, which is of particular ease in multicenter clinical trials.[47] A disadvantage of this assay is the labour intensive sample preparation as well as the requirement for a trained technician.

2.6.3 Clinical Utility

A recent study revealed that the flowcytometric VASP is one of the most suitable tests for determining biological activity of clopidogrel, by showing an excellent correlation between the test-results and the bioavailability of the active metabolite of clopidogrel.[32]

Furthermore, a relation between high on-treatment platelet reactivity, as assessed with VASP, and clinical outcome, has been described. First, Barragan and coworkers performed a prospective evaluation using the VASP-assay in order to detect patients at risk.[48] Among 1684 consecutive stented patients, 16 presenting with ST were compared with 30 stented patients free from ST. A significant difference was observed between patients with and without ST (63.3±9.6% vs. 39.8±10.9%, p<0.0001). However, this finding does not allow us to conclude whether high on-treatment platelet reactivity is a predisposing factor or a consequence of ST.

Frere and colleagues were the first to observe the ability of VASP to identify patients at higher risk of thrombotic events. In 195 patients with NSTEMI undergoing PCI, the VASP PRI was analyzed after a clopidogrel loading dose of 600 mg.[13] The primary end-point was recurrent ischemic events at 30-days follow-up. ROC-curve analysis identified an optimal cut-off value of 53% PRI. Patients above this cut-off, exhibiting high on-treatment platelet reactivity, had significantly more often a CV event as compared to patients below the cut-off (12.3% vs.1.1%, p<0.001)

The predictive value of the VASP assay was then gauged by Bonello and colleagues, who described whether a high PRI influences clinical outcome in a population undergoing PCI with stenting (n=144. VASP was performed 24 hours after a 300 mg loading dose.[15] Patients were divided into quintiles according to PRI. Patients in the lowest quintiles had a significantly lower risk of CV death, ACS, stroke and repeated revascularization as compared with the higher quintiles (0% vs.2.1%, p<0.01). ROC-curve analysis revealed an optimal cut-off value of 50% PRI, with a negative predictive value of 100%.

Recently Siller-Matula and colleagues compared the VASP-assay with the MEA in 416 patients with CAD undergoing PCI.[21] The primary endpoint was ST at 6-months follow-up.
ROC-curve analysis demonstrated that VASP is able to identify patients at higher risk of (definite and probable) ST, using a cut-off value of 42%. Patients exhibiting high on-treatment platelet reactivity had a 1.6-fold higher risk of ST.

2.7 IMPACT-R

2.7.1. Description of the test

The IMPACT-R device (DiaMed, Cresier, Switzerland) is based on the cone and plate(let) analyzer technology[49] and intents to mimic the interaction of platelets with the subendothelium under flow conditions with similar shear forces.

Citrated whole blood samples are pre-stimulated with a sub-optimal concentration ADP (1.38µmol/L) and gently mixed (10 RPM) for 1 minute. Pre-stimulation with ADP leads to the formation of micro-aggregates in patients not using clopidogrel or in whom clopidogrel does not effectively inhibit platelet function. These micro-aggregated platelets temporarily lose their adhesive properties. Aliquots of the ADP-pre-incubated whole blood sample (130 µl) are transferred to a polystyrene well and subjected to shear (1800 s^{-1} for 2 min) using a rotating cone. Under these test conditions, vWF and fibrinogen are instantly immobilized on the polystyrene surface, serving as a

substrate for platelet adhesion and subsequent aggregation. The wells are washed and stained with May-Grunwald stain and analyzed with an inverted light microscope that is connected to an image analysis system. Platelet adhesion and aggregation on the surface are evaluated by examining the percentage of total area covered with platelet designated as surface coverage (SC) and the average size of surface-bound objects (i.e. platelet aggregates). The percentage SC is inversely correlated with the magnitude of ADP-induced platelet activation.

2.7.2 Strengths and drawbacks

The IMPACT-R is based on shear stress. This is potentially advantageous because shear is of utmost importance in the pathophysiology of thrombus formation. The method needs a low sample whole blood volume. However, the accuracy of the test might be hampered because the device requires multiple sample preparation proceedings. Furthermore, because of this extensive sample handling it cannot be considered as a true point-of-care assay.

2.7.3. Clinical Utility

Matetzky et al. evaluated the response to clopidogrel therapy among 60 patients with a ST-elevation myocardial infarction (STEMI) undergoing PCI with stenting. Platelet reactivity was assessed using the IMPACT-R and patients were stratified into quartiles according to the magnitude of platelet reactivity. Patients in the highest quartile of platelet reactivity were considered to have high-on treatment platelet reactivity. At six-month follow-up, eight CV events occurred. Seven of these events (88%) were observed in those patients exhibiting high-on treatment platelet reactivity. Since the sample-size of this study is relatively small, it does not allow definite conclusions.

It is presumed that platelet function testing under 'physiological' high shear conditions reflects the physiological milieu more precisely than ex vivo aggregation-based platelet function tests. However, a recent study has demonstrated that the magnitude of platelet reactivity as measured by the IMPACT-R ADP are inversely correlated with the active thiol metabolite of clopidogrel (r= -0.48, *p=0.03*) and is therefore not suitable to determine the in vivo bioavailability of the active metabolite of clopidogrel.[32] Furthermore, the POPular study recently demonstrated in a large cohort of patients that the IMPACT-R was not able to predict the combined endpoint of death, MI, ST and stroke (7.5% vs.9.8%, p=0.21 using IMPACT-R [n=910] and 7.9% vs.8.6%, p=0.68 using IMPACT-R ADP [n=905]), nor its single components.[20]

2.8 Different methods of platelet function testing

Thromboelastography (TEG), designed for evaluating blood coagulation during surgical procedures, was first described more 50 years ago[50] and has recently been modified to a more platelet-specific test in the form of the TEG Platelet Mapping system (Haemoscope, Niles, IL).[51] This assay is able to assess platelet function and coagulation simultaneously. However, to date no data concerning the predictive accuracy of the TEG Platelet Mapping system are available.

Two new instruments have been developed recently; the PlaCor PRT time (PlaCor, Plymouth, USA) and the ThromboGuide Platelet Aggregation Analyzer (Thrombovision, Houston USA).

The PlaCor PRT time, first introduced at the fifth International Platelet Symposium (Platelets 2008), is a point-of-care device designed to measure platelet function in non-anticoagulated blood obtained from a fingerprick. The assay measures the time to platelet thrombus formation in response to shear stress ($1600s^{-1}$), induced by pumping blood in alternate directions through a constriction/coil in two channels. Average time to cessation of flow is reported as PRT time. A small study has demonstrated a good in-laboratory correlation between the PlaCor PRT time and ADP-induced whole blood aggregometry ($R_{pearson}$=-0.74). However, whether this assay is able to demonstrate a relationship between HPR and clinical outcome remains to be established.

The ThromboGuide Platelet Aggregation Analyzer (T-Guide), which is currently under development, measures platelet aggregation using a light scattering technology. The T-Guide is based upon the assumption that red blood cells scatter light (background or baseline) and platelet aggregates cause perturbations that alter the recorded backscatter. The number of platelet aggregates formed in response to an agonist reflects the extent of the antiplatelet drug's effect. This system has currently three types of single-use, disposable cartridges that can be used to monitor different antiplatelet drugs: aspirin (using arachidonic acid as an agonist), P2Y12 (using ADP as an agonist) and GP IIb/IIIa assay (using TRAP as an agonist). T-Guide has not been evaluated yet.

3 HOW TO HANDLE HIGH ON-TREATMENT PLATELET REACTIVITY?

3.1 Individualizing therapy

Thus far, little data are available concerning the benefit of tailoring therapy based on the results of platelet function testing. Therefore, the correct treatment-if any-of high on-treatment platelet reactivity remains unknown.

Nonetheless, the American College of Cardiology (ACC), the American Heart Association (AHA), and the Society for Cardiovascular Angiography and Interventions (SCAI) recommend the following (Class IIb, level of evidence C): "In patients in whom stent thrombosis may be catastrophic or lethal (unprotected left main, bifurcating left main, or last patent coronary vessel), platelet aggregation studies may be considered and the dose of clopidogrel increased to 150 mg per day if <50% inhibition of platelet aggregation is demonstrated".[52]

Three small studies indeed suggest that individualizing therapy based on platelet function might improve outcome. Bonello and coworkers investigated the effect of VASP-guided adjustment of the clopidogrel loading dose in patients exhibiting high on-treatment platelet reactivity undergoing PCI (n=162).[26] In this study, patients exhibiting high on-treatment platelet reactivity (defined as PRI>50%) were randomized to a control group or to a VASP-guided group, in which they received additional doses of 600 mg clopidogrel. The use of up to three additional boluses significantly decreased the PRI (69.3±10% to 37.6±13.8%, $p < 0.001$). Moreover, in the VASP-guided group the incidence of major adverse cardiac events at one month follow-up was significantly lower

as compared with the control group (0% vs. 10%; p=0.007).

This finding was confirmed in a larger randomized clinical trial including 429 patients exhibiting high on-treatment platelet reactivity undergoing PCI. Patients were randomized to either a control group or a VASP-guided group.[27] The primary endpoint was the rate of stent thrombosis at 1 month. The incidence of stent thrombosis was significantly lower in the VASP-guided group as compared to the control group (0.5% vs. 4.2%), p <0.01 as well as the rate of major adverse cardiovascular events (0.5% vs 8.9%, p <0.001).

In a recently performed trial, 215 patients undergoing drug-eluting stent (DES) implantation for unprotected left main disease (ULMD) were included.[53] Platelet function was assessed using LTA 10 µmol/L ADP and high on-treatment platelet reactivity was defined as > 70% of aggregation. All patients were prescribed to a maintenance dose of aspirin (325mg) and clopidogrel (75mg), but patients exhibiting high on-treatment platelet reactivity were prescribed 150 mg clopidogrel daily or were shifted to ticlopidine (500 mg daily) prior to PCI. The effect of this change in therapy on the magnitude of platelet reactivity was not tested. Despite the double dose, cardiac mortality was significantly higher in patients exhibiting high on-treatment platelet reactivity (28.3% vs.8.0%, p=0.005) as well as the incidence of ST (16.0% vs. 4.2%, p=0.021). Patients exhibiting high on-treatment platelet reactivity had an almost four-fold higher risk of cardiac death (HR= 3.8; 95% CI: 1.4-10.5; *p*=0.010) and ST(HR=3.7; 95%CI: 1.1-12.1, p=0.031). This is in line with the observation that even at a higher maintenance dose of 150 mg daily there is still a large variability in the degree of platelet inhibition.[54] Potential promising strategies to improve outcome based on platelet function testing, might thus include switching to more potent P2Y12-receptor antagonists.

Recently, Pena and colleagues reported seven cases of early definite ST in patients exhibiting high on-treatment platelet reactivity.[55] In the weeks following ST, all patients received a stepwise increase in clopidogrel maintenance dose. A dose up to 300 mg daily only slightly reduced platelet reactivity. Four patients finally switched to prasugrel, which led to a significant reduction of on-treatment platelet reactivity in all four.

3.2 New P2Y12-receptor antagonists

Several previous studies have shown advantageous effects of more potent P2Y12-receptor-antagonists in patients with acute coronary syndromes undergoing PCI.[56,57] The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction (TRITON–TIMI) 38 demonstrated that the use of prasugrel compared to clopidogrel led to a further reduction of the composite endpoint containing cardiovascular death, MI, or stroke (absolute risk reduction of 2.2%).[56] A counterpart of prasugrel was the increase in the occurrence of bleeding in the prasugrel-treated patients (2.4% vs. 1.8% major bleeding, p=0.03 and 1.4% vs. 0.9%;
p=0.01 life-threatening bleeding).

Furthermore, The Study of Platelet Inhibition and Patient Outcomes (PLATO) showed that

treatment with ticagrelor resulted in an absolute risk reduction of 1.9% of the composite endpoint (death from vascular causes, myocardial infarction [MI], or stroke) as compared to clopidogrel, without an increase in major bleeding.[57]

3.3 GPIIb/IIIa-antagonists

Another way of individualizing therapy is the addition of GPIIb/IIIa-receptor blockers.[58,59]
Cuisset and colleagues demonstrated in a small population of 149 patients with high on-treatment platelet reactivity undergoing elective PCI, that the addition of abciximab significantly reduced CV events (40% vs. 19%, p=0.006), without an increase in major bleeding.[58]

In addition, Valgimigli and coworkers explored in the Double-Blind, Prospective, Randomized Tailoring Treatment With Tirofiban in Patients Showing Resistance to Aspirin and/or Resistance to Clopidogrel Study (3T/2R Study) whether patients exhibiting high on-treatment platelet reactivity would benefit from an intensified platelet inhibition by use of tirofiban.[59] The study enrolled 263 patients undergoing elective coronary angioplasty for stable or low-risk unstable CAD. High on-treatment platelet reactivity was assessed using the VerifyNow P2Y12 cartridge. Patients with high on-treatment platelet reactivity were randomly assigned to receive tirofiban (n=132) or placebo (n=131) on top of standard aspirin and clopidogrel therapy. The primary end point, the incidence of periprocedural myocardial infarction defined as an elevation of Troponin I or T at least three times the upper limit of normal within 48 hours after the procedure, occurred significantly more frequently in the placebo group as compared to the tirofiban group (35.1% vs 20.4%, p=0.009). The 30-days rate of MACE was also significantly higher in the placebo group (10.7% vs. 3.8%, p=0.031). The incidence of bleeding was low and did not differ significantly between the two groups.

4. THE DRAWBACK OF ENHANCED PLATELET INHIBITION

The logical counterpart of efficient platelet inhibition is the risk of bleeding complications. Whereas the evidence that high on-treatment platelet reactivity strongly influences the occurrence of thrombo-ischemic events in PCI-patients is overwhelming; data concerning the association between platelet reactivity and bleeding are limited.

Cuisset and coworkers were the first to demonstrate a relation between enhanced platelet inhibition and the occurrence of post-discharge non-CABG-related bleeding in a large cohort of patients presenting with NSTEMI undergoing PCI (n=597).[60] LTA and VASP were used to assess platelet reactivity. Patients with bleeding had a significantly lower magnitude of platelet reactivity as assessed by both LTA (43±4% vs. 56±19%, p=0.002) and VASP (43±4% vs. 54±23%, p=0.04) and after stratification into quartiles based on the magnitude of platelet reactivity, patients in the lowest quartile of ADP-induced aggregation (<40%) were considered as exhibiting an enhanced inhibition of platelet function. The risk of Thrombolysis in Myocardial Infarction (TIMI) major and minor bleeding was significantly higher in the first quartile as compared to the other quartiles (6.6 vs. 1.4%, p=0.001).

Recently, Sibbing et al. assessed the impact of platelet inhibition on bleeding risk in clopidogrel treated patients undergoing PCI (n=2533).[61] ADP-induced platelet aggregation was assessed on the Multiplate® analyzer. ROC-curve analysis was performed to determine an optimal cut-off value based on the primary endpoint (in-hospital TIMI major bleeding). This study demonstrated that an enhanced platelet inhibition (defined as <188 AU*min) caused by clopidogrel is indepently associated with a higher risk of major bleeding ($OR_{adjusted}$=3.5; 95% CI: 1.6-7.3, p=0.001).

These studies are the first suggesting that measuring platelet function might be the solution to define a therapeutic window between bleeding and thrombotic events.

5. CONCLUSION

The individual response to the "one-size fits all" dosing of antiplatelet therapy is highly variable resulting in a high on-treatment platelet reactivity status in a substantial number of patients. Moreover, numerous studies have established a clear link between high on-treatment platelet reactivity measured by multiple methods and the occurrence of atherothrombotic events. However, due to a lack of consensus on the optimal method to measure high on-treatment platelet reactivity, platelet function testing has not been widely implemented in current clinical practice. The VerifyNow® assays and the Multiplate® system seem to be the most promising easy-to-use platelet function tests but any evidence demonstrating that the adjustment of antiplatelet therapy on the basis of the results of these tests improves clinical outcomes is lacking **(Table 3)**. Until the results of these "tailoring studies" are available, the routine use of platelet function testing in clinical practice is not recommended.

6. AIMS AND OUTLINE OF THIS THESIS

Although a growing body of evidence demonstrates the promising potential of platelet function tests in predicting atherothrombotic events post stenting, platelet function testing should not be used beyond clinical trials, largely because it is not clear if modifications in antiplatelet therapy based on the results of platelet function tests can reduce the occurrence of atherothrombotic events. Class IIB recommendations from the ACC/AHA state that platelet aggregation studies are warranted in patients undergoing PCI who are at risk of sub-acute stent thrombosis, with the option to increase their maintenance dose of clopidogrel from 75 mg/day to 150 mg/day in order to suppress platelet reactivity below 50%.[52] However, the recommendations give no indication regarding which assay should be used in this regard and which level of on-treatment platelet reactivity is the most adequate to identify patients at high risk of atherothrombotic events. Multiple studies have identified as many cut-off values **(Table 4**). Both quartile approaches as well as ROC curve analysis have been used to determine these optimal cut-off levels to discriminate patients at a higher ischemic risk. Of these methods, the ROC curve analysis might be considered the more valuable approach since these cut-off values are derived by an accepted statistical test and moreover are associated with the lowest false negative and false positive rates. Owing to the controversy surrounding this recommendation,

Tabel 3: Studies evaluating the benefit of tailoring therapy based on the VerifyNow

	Size	Setting	Cut-off value	Primary Endpoint	Results available
GRAVITAS	2783	Patients exhibiting HPR undergoing PCI with DES implantation *Fixed arm* 450 mg loading dose, 75 mg clopidogrel maintenance **Dose-adjusted arm** 900 mg loading dose, 150 mg clopidogrel maintenance **Extra control group: Patients without HPR** 450 mg clopidogrel loading dose, 75 mg maintenance	PRU>230	Time to CV death, non-fatal MI or definite/probable ST	2010
DANTE	422	Patients exhibiting HPR, presenting with UA/NSTEMI, undergoing PCI with stenting *Fixed arm* 75 mg clopidogrel maintenance therapy **Dose-adjusted arm** 150 mg clopidogrel maintenance therapy	PRU>240	6-month/12-month MACE (CV death, nonfatal MI, TLR)	2011
ARCTIC	2500	All patients undergoing PCI with DES implantation. Patients exhibiting HPR will be randomized to either conventional or double dose *Fixed arm* 75 mg clopidogrel maintenance therapy **Dose-adjusted arm** 150 mg clopidogrel maintenance therapy	PRU>235	12-month Composite of death, MI, ST, stroke, urgent revascularization	2011
TRIGGER-PCI	2150	Patients exhibiting HPR undergoing elective PCI with DES implantation *Fixed arm* 75 mg clopidogrel maintenance therapy **Dose-adjusted arm** 60 mg prasugrel loading dose, 10 mg maintenance therapy	PRU>208	6-month Time to CV-death or MI	2011

HPR=high on-treatment platelet reactivity, PCI=percutaneous coronary intervention, DES=drug eluting stent implantation, PRU= P2Y12 reaction units, CV=cardiovascular, MI=myocardial infarction, ST=stent thrombosis, UAP=unstable angina, NSTEMI=non-ST-elevation myocardial infarction, TLR=target lesion revascularization

the general consensus is that platelet function testing and treatment alterations based on these tests are not recommended for routine clinical practice.[62]

This thesis addresses these issues and aims to provide insight into platelet function testing and its clinical applicability. **Part I** of this thesis describes the ability of multiple platelet function tests in predicting clinical outcome in patients on dual antiplatelet therapy undergoing elective coronary stent implantation. First, various parameters of classic light transmittance aggregometry (LTA) are

Table 4: Cut-off values of multiple platelet function tests for the occurrence of atherothrombotic events post-PCI

Platelet Function Test	Cut-off Value
Light transmittance Aggregometry	
5 µmol/L ADP	46% aggregation[60], 42.9% aggregation[19]
20 µmol/L ADP	59% aggregation[60] , 64.5% aggregation[19]
VerifyNow	235 PRU[15], 236 PRU[19], 240 PRU[16]
Multiplate	
ADP-test	468 AU[18]
HS ADP-test	54 U[20]
Plateletworks	80.5% aggregation[19]
PFA-100 COL/ADP	118 seconds, 116 seconds[19]
VASP	48% PRI[62], 50% PRI[11]
IMPACT-R	2.8% SC[61], 3.0% SC[19]

LTA=light transmittance aggregometry, ADP=adenosinediphophate, PRU= P2Y12-reaction units, HS= high sensitive, PFA=platelet function analyzer, VASP= vasodilator-stimulated phosphoprotein, PRI= platelet reactivity index

compared (chapter 1). Second, platelet function tests assessing the efficacy of clopidogrel (chapter 2 and 3), aspirin (chapter 4) or both (chapter 5) are evaluated.

In **part II** the effect of several patient characteristics on the magnitude of platelet reactivity and clinical outcome is described. The effect of diabetes mellitus (chapter 6), renal failure (chapter 7) and gender (chapter 8) on platelet reactivity, the incidence of high on-treatment platelet reactivity and subsequent clinical outcome is investigated. In chapter 9 a population presenting with ST-elevation myocardial infarction is described and chapter 10 studies a population that suffered from stent thrombosis. Chapter 11 evaluates three subgroups of patients having a higher risk of bleeding. In chapter 12 we discuss the possibilities for tailoring therapy and describe the new options in antiplatelet therapy.

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Chapter 1a

Standardization of light transmittance aggregometry

Do not adjust the platelet count in light transmittance aggregometry when predicting thrombotic events after PCI

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Dual antiplatelet therapy with aspirin and clopidogrel reduces thrombotic complications in patients undergoing percutaneous coronary intervention (PCI). [1,2] A growing body of evidence demonstrates that the efficacy of dual antiplatelet therapy is highly variable and that high on-treatment platelet reactivity (HPR) is associated with the occurrence of atherothrombotic events.[3-6]

"Classical" light transmittance aggregometry (LTA) is still considered to be the gold standard to quantify the magnitude of on-treatment platelet reactivity, but it remains poorly standardized and various parameters are used by different laboratories to determine the magnitude of platelet reactivity.[7] The adjustment of the platelet-rich plasma (PRP) to achieve a platelet count of 250.000/μL has been proposed to standardize LTA in patients with bleeding diathesis. Since no clinical endpoint studies have previously determined whether LTA using either "native" or adjusted (standardization of the platelet count to 250.000 /μL) PRP is a better predictor of adverse events, it remains controversial whether adjustment of the platelet count is necessary for the monitoring of antiplatelet therapy in cardiovascular patients on aspirin and clopidogrel. Therefore, the aim of the present study was to evaluate the value of LTA in predicting atherothrombotic events, using both "native" and adjusted PRP.

A prospective cohort study of consecutive patients undergoing elective PCI with stent-implantation was performed.[4] All patients received clopidogrel treatment before PCI and all patients were on aspirin at a dose of 80 to 100 mg daily ≥10days, unless they were on long-term anticoagulation with coumadins. The primary endpoint was defined as a composite of all-cause death, non-fatal myocardial infarction, definite stent thrombosis and ischemic stroke.

Light transmittance aggregometry (LTA) was assessed on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Samples were centrifuged for 10 minutes at 150*g* to obtain native PRP. Platelet poor plasma (PPP) was obtained by centrifuging the remaining sample at 1500*g* for 10 minutes. Half of the amount of native PRP was adjusted (with PPP) to achieve a calculated platelet count of 250.000/μL. Patients with a platelet count < 300.000/μl in PRP before adjustment were excluded. PPP was set as 100% aggregation and maximal (peak) platelet aggregation (%) induced by ADP in a final concentration of 20 μmol/L was measured in PRP. To evaluate LTA's ability to discriminate between patients with and without atherothrombotic events one-year post-PCI, a receiver-operator characteristic (ROC) curve analysis was performed for both adjusted and non-adjusted PRP. The optimal cut-off level was calculated by determining the smallest distance between the ROC-curve and the upper left corner of the graph (i.e. the point with the highest sensitivity as well as specificity). Patients above the optimal cut-off were considered to exhibit HPR. The predictability of the parameters, *i.e.* the ability of the test to correctly classify those with and without atherothrombotic event, was expressed as area under the curve (AUC).

LTA induced by 20 μmol/L ADP was performed in 1051 patients undergoing elective PCI with stent implantation. Of these, 753 patients had a platelet count in native PRP >300.000/μL. Owing to logistic demands or a low volume of PRP, PRP-samples of 692 patients were adjusted to a platelet count of 250.000/μL. The latter cohort comprised the present analysis. Platelet count in native PRP

was 418.600/µL ± 92.900 as compared to 260.300/µL ± 23.000 in adjusted PRP. The magnitude of on-treatment platelet reactivity was significantly higher when native PRP was used compared to adjusted PRP (58.2% ± 14.0 vs. 49.2% ± 16.4, p<0.0001). In addition, the ROC curve derived cut-off value was higher when native PRP was used as compared to adjusted PRP (67.0% vs. 58.7%).

At one-year follow-up the primary endpoint occurred more frequently in patients with HPR as compared to patients without HPR, using both native PRP (30/200 [15.0%] vs. 33/492[6.7%]; OR 2.45; 95%-CI=1.45-4.15, p=0.001) as well as adjusted PRP (30/243 [12.3%] vs.33/449 [7.3%]; OR 1.78; 95%-CI=1.05-2.99, p=0.04) (**figure 1**). In addition, the predictability was similar in LTA using either native or adjusted PRP (AUC=0.59; 95%-CI=0.52-0.66 for both). The negative predictive value (NPV) of both is high and the positive predictive value (PPV) is low (NPV=93.3.% and PPV=15.0% using native PRP; NPV= 92.7.% and PPV=12.3% using adjusted PRP) , which is in agreement with other platelet function studies linked to clinical outcome.

Although LTA is still regarded as the 'gold standard' method, this technique is poorly standardized since no external quality assessment is available and no standard platelet function testing protocol has been unanimously adopted. Throughout the last two decades, several attempts have been introduced to increase the between-centre comparability of LTA by standardization of 1) the platelet storage temperature prior to testing, 2) stirring rate, 3) centrifugation speed to obtain PRP and PPP, 4) agonist sources and 5) the adjustment of the platelet count in platelet rich plasma to a standard count.[7-13] However, the frequently used procedure of adjusting the platelet number in PRP is cumbersome, may affect platelet activation and has been questioned since it does not reflect the platelet function *in vivo*.[7,14,15] Moreover, the avoidance of the time-consuming step of platelet count adjustment would make LTA easier accessible for the routine monitoring of antiplatelet therapy in clinical practice.[14]

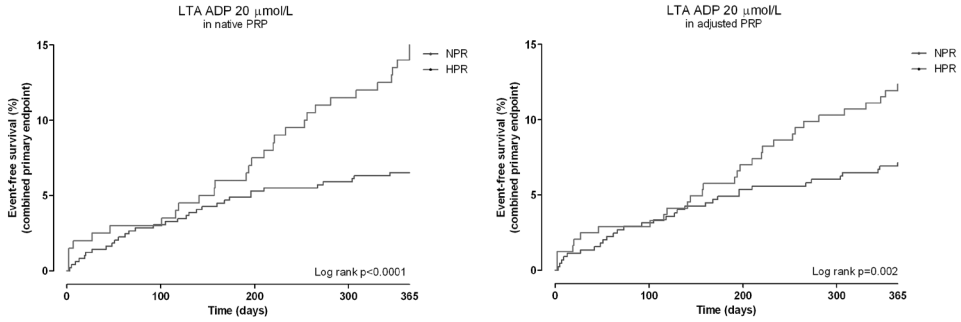

Figure 1: Kaplan-Meier Analysis

Kaplan-Meier analysis for the event rate for the combined primary endpoint in patients with and without high on-treatment platelet reactivity as measured in both native and adjusted PRP

Native PRP = LTA performed using native platelet rich plasma; Adjusted PRP= LTA performed using platelet count-adjusted to a calculated platelet count of 250.000/µL platelet rich plasma (PRP)

HPR = high on-treatment platelet reactivity according to the defined cut-off (i.e. >67.0% aggregation in native PRP and >58.7% in platelet count adjusted PRP) NPR = normal on-treatment platelet reactivity according to the defined cut-off

In the present study, the predictive value of both native and platelet count adjusted PRP for the occurrence of adverse atherothrombotic events was evaluated. Although the ROC-based cut-off value to segregate patients with and without HPR was considerably lower when PRP was adjusted, both procedures share equal predictability for adverse clinical outcome. Thus, the adjustment of platelet count does not provide additional information. Light transmittance aggregometry using native PRP is easier to perform and has a similar accuracy in predicting atherothrombotic events. Therefore we advise not to adjust the platelet count in platelet rich plasma when predicting thrombotic events after PCI.

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Chapter 1b

Standardization of light transmittance aggregometry

Both peak and late aggregation are capable to identify patients at risk for atherothrombotic events

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The addition of clopidogrel to lifelong aspirin treatment reduces atherothrombotic events in patients with acute coronary syndromes and those undergoing percutaneous coronary intervention (PCI) with stent implantation.[1;2] However, the individual response to clopidogrel is unpredictable resulting in high on-treatment platelet reactivity (HPR) in a substantial number of patients. Consistent findings across multiple investigations point out to a strong relationship between HPR and the occurrence of atherothrombotic events[3;4] and multiple studies have used 'gold standard' light transmittance aggregometry (LTA) to demonstrate this association.[5-9]

Clopidogrel targets the P2Y12-receptor that plays an important role in thrombus formation and stabilization.[10-13] Recent studies have demonstrated that the evaluation of late aggregation instead of the more commonly used maximal ('peak') level of aggregation may be more representative of P2Y12 receptor signaling[15;16] and, of even more importance, late aggregation is also associated with adverse clinical outcome.(7) Although contrasting results have been reported regarding the timing of assessment (peak vs. late platelet aggregation) there is currently no data based on clinical endpoints.[15;16] The POPular- study (*The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI)* demonstrated that peak aggregation was able to predict the occurrence of an adverse cardiovascular event in patients undergoing elective PCI with stent implantation.[9] The present sub-analysis, an extension on this study, aimed to compare the capability of LTA to predict atherothrombotic events using either peak or late aggregation.

A prospective cohort study of 1069 consecutive patients undergoing elective PCI with stent-implantation was performed.[9] All patients received optimal clopidogrel treatment prior to PCI (defined as a maintenance of 75 mg daily therapy for >5 days or a loading dose of 300 mg at least 24h before PCI or 600 mg at least 4 hours before PCI. All patients used aspirin at a dose of 80 to 100 mg daily ≥10days, unless they were on long-term anticoagulation with coumarin derivates. The primary endpoint was defined as a composite of all-cause death, non-fatal myocardial infarction, definite stent thrombosis and ischemic stroke.

Light transmittance aggregometry (LTA) was performed in non-adjusted platelet-rich plasma (PRP) on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Whole blood samples (collected in 3.2% citrated tubes) were centrifuged for 10 minutes at 150*g* to obtain native PRP. Platelet poor plasma (PPP) was obtained by centrifuging the remaining sample at 1500*g* for 10 minutes. PRP samples were kept at room temperature. After addition of ADP in a final concentration of 20 µmol/L, the magnitude of aggregation was monitored for 10 minutes. All tracings were inspected by a clinical chemist and a cardiologist experienced in evaluating platelet function. PPP was set as 100% aggregation and both peak aggregation (%), which is automatically reported by the aggregometer, as well as late platelet aggregation (%) were measured. According to our protocol, late aggregation is measured 360s after agonist addition. Due to the (partly) reversible character of aggregation tracings in patients on clopidogrel, peak aggregation is normally reached before 360 seconds.

A receiver-operator characteristic (ROC) curve analysis was calculated for both peak and late

aggregation in order to evaluate LTA's ability to discriminate between patients with and without primary endpoint at one-year follow-up. The optimal cut-off level was established by determining the smallest distance between the ROC-curve and the upper left corner of the graph (*i.e.* the point with the highest sensitivity as well as specificity). Patients above the optimal cut-off level were considered to exhibit HPR. The predictability of the parameters, *i.e.* the ability of the test to correctly classify those with and without atherothrombotic event, was expressed as area under the curve (AUC).

Owing to irregularities in supply or technical failure, in a total of 18 patients no LTA was performed. As a consequence twenty µmol/L ADP-induced LTA was available in 1051 patients. As expected, the magnitude of on-treatment platelet reactivity was significantly higher using peak as compared to late aggregation (57.7% ± 14.7 vs. 41.1% ±24.2, p<0.0001) and the cut-off value to identify patients at risk was higher when peak aggregation was established as compared to late aggregation (64.5% vs. 46.2%). Using peak aggregation 392/1051 (37.3%) patients exhibited high on-treatment platelet reactivity and 511/1051 (48.6%) had high on-treatment platelet reactivity using late aggregation. At one-year follow-up, the primary endpoint occurred more frequently in patients with HPR as compared to patients without HPR, expressed as peak (47/392 [12.0%] vs. 41/659 [6.2%]; OR 2.05; 95%-CI=1.32-3.19, p=0.001) or late aggregation (57/511 [11.2%] vs. 31/540 [5.7%]; OR 2.06; 95%-CI=1.31-3.25, p=0.002) **(figure 1**). After adjustment for potential confounders known to influence platelet reactivity age, BMI, hypertension, clopidogrel loading dose, hemoglobin and an impaired left ventricular ejection fraction, the Odds ratios remained statistically significant: OR 2.06; 95%-CI=1.29-3.28, p=0.002 for peak and OR 2.07; 95%-CI=1.28-3.35, p=0.003 for late aggregation.

However, the predictability, represented by the areas under the curve (AUC), was similar when LTA was expressed as either peak or late aggregation (AUC=0.62; 95%-CI=0.56-0.67 for peak and

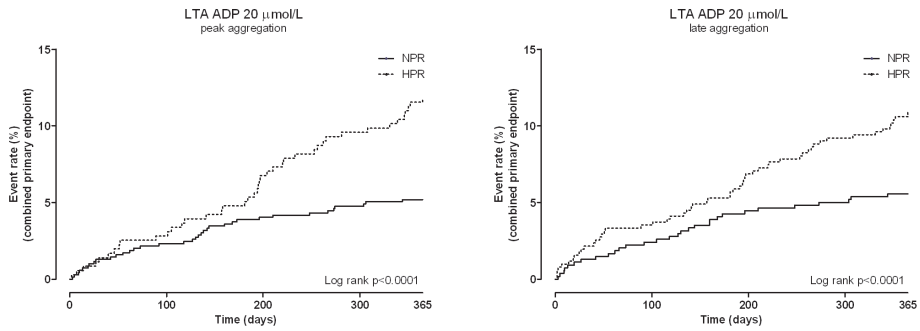

Figure 1: Kaplan-Meier Analysis

Kaplan Meier analysis for the event rate for the combined primary endpoint in patients with and without high on-treatment platelet reactivity according to both peak and late aggregation

Peak aggregation= maximal aggregation; Late aggregation= final aggregation (360 seconds after agonist addition) HPR = percentage of patients exhibiting high on-treatment platelet reactivity according to the defined cut-off (i.e. >64.5% peak aggregation and >46.2% late aggregation) NPR = percentage of patients exhibiting normal on-treatment platelet reactivity according to the defined cut-off

AUC=0.62; 95%-CI=0.56-0.68 for late aggregation). In addition, the two platelet function measures demonstrated substantial agreement regarding the classification of individuals into HPR and normal responder; the percentage agreement was 86.4% and the Kappa=0.73. The negative predictive value (NPV) of both is high and the positive predictive value (PPV) is low (NPV=93.8.% and PPV=12.0% using peak aggregation; NPV= 94.3.% and PPV=11.2% using late aggregation).

One of the questions regarding the measure of efficacy of clopidogrel relates to the timing of the evaluation of the ADP-induced aggregation curve in clopidogrel treated patients.[17-21] The peak level of ADP-induced platelet aggregation is the most frequently used measure of the aggregation curve, but some investigators have proposed that late aggregation might be a more appropriate measure to estimate the effectiveness of clopidogrel therapy. The rationale behind this is the fact that the active metabolite of clopidogrel selectively inhibits ADP binding to the P2Y12-receptor (responsible for the stabilization of platelet aggregation)[22] but not the P2Y1-receptor (responsible for rapid platelet shape change, phospholipase-C-activation and calcium release from internal stores).[13;23]

Our study demonstrated that, although the ROC-based cut-off value to identify patients at risk of atherothrombotic events was substantially higher for maximal aggregation as compared to peak aggregation, both parameters shared equivalent predictability. Thus, peak and late aggregation are able to identify patients at risk with equivalent accuracy, indicating that peak and late aggregation might be interchangeable. Therefore, there is no reason to replace peak by late aggregation. However, the predictability of both peak and late aggregation was only modest. In addition, there is only preliminary data concerning the benefit of tailoring therapy based on the results of platelet function testing. Therefore, in line with a recently published consensus opinion on platelet function testing[24], we do not recommend to measure platelet reactivity routinely in daily clinical practice."

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Chapter 2

Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation

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ABSTRACT

Context: High on-treatment platelet reactivity (HPR) is associated with atherothrombotic events following coronary stent implantation.

Objective: To evaluate the capability of multiple platelet function tests to predict clinical outcome.

Design, Setting and patients: Prospective, observational, single-center cohort study of 1069 consecutive patients on clopidogrel undergoing elective coronary stent implantation between December 2005 and December 2007. On-treatment platelet reactivity was measured in parallel by light transmittance aggregometry (LTA), the VerifyNow® P2Y12 assay, the Plateletworks® assay, the IMPACT-R and the PFA-100® System (with the Dade® PFA Collagen/ADP Cartridge and INNOVANCE® PFA P2Y*). Cut-off values for HPR were established by receiver-operator characteristic (ROC) curve analysis.

Main Outcome Measurement: The primary endpoint was defined as a composite of all-cause death, non-fatal acute myocardial infarction, stent thrombosis and ischemic stroke. The primary safety endpoint included TIMI major and minor bleeding.

Results: Kaplan-Meijer analysis demonstrated that at one-year follow-up, the primary endpoint occurred more frequently in patients with HPR when assessed by LTA (52[11.7%{95%-CI=8.9-15.0%}] vs 36[6.0%[{95%-CI=4.2-8.2%}], p=0.0009 [n=1049]) ,VerifyNow® (54[13.3%{95%-CI=10.2-17.0%}] vs 37[5.7%{95%-CI=4.1-7.8%}], p<0.0001 [n=1052)]), Plateletworks® (33[12.6%{95%-CI=8.8-17.2%}] vs 21[6.1%{95%-CI3.8-9.2%}], p=0.005 [n=606])-and INNOVANCE® PFA P2Y* (18[12.2%{95%-CI=7.4-18.6%}] vs 28[6.3%{95%-CI=4.3-8.9%}, p=0.02 [n=588]).

ROC-curve analysis demonstrated that LTA (AUC=0.63;95%-CI:0.58-0.68), VerifyNow® (AUC=0.62;95%-CI:0.57-0.67) and Plateletworks® (AUC=0.61; 95%-CI:0.53-0.69) were able to discriminate between patients with and without primary endpoint. The IMPACT-R (n=905) and the Siemens® PFA Collagen/ADP (n=812) were unable to discriminate between patients with and without the primary endpoint at one-year follow-up.

None of the platelet function tests were able to identify patients at risk for bleeding.

Conclusion: Of the platelet function tests assessed only LTA, VerifyNow®, Plateletworks® and INNOVANCE® PFA P2Y* were significantly associated with the primary endpoint. However, the predictability of these four tests was only modest. None of the tests provided accurate prognostic information to identify patients at higher risk of bleeding. Thus, the POPular-study does not support the use of platelet function testing to guide clinical practice in a low-risk population of patients undergoing elective PCI.

INTRODUCTION

Dual antiplatelet therapy with aspirin and clopidogrel reduces atherothrombotic complications in patients undergoing percutaneous coronary intervention (PCI) with stenting.[1,2] However, the individual response to dual antiplatelet therapy is not uniform and consistent findings across multiple investigations support the association between a lower degree of platelet inhibition, a high on-treatment platelet reactivity (HPR), and the occurrence of atherothrombotic events.[3-10]

The major drawbacks of these previous investigations are the relatively small sample size of the studied populations and the fact that on-treatment platelet reactivity was evaluated by only one platelet function test per study. There is currently no consensus regarding the most appropriate method to quantify the magnitude of on-treatment platelet reactivity. Therefore, the aim of *The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI (the POPular-study)* was to evaluate the ability of multiple platelet function tests in predicting atherothrombotic events, including stent thrombosis, in clopidogrel pre-treated patients undergoing PCI with stent implantation.

METHODS

Study Population

Consecutive patients with established coronary artery disease scheduled for elective PCI with stent implantation were included in this study. All patients received optimal clopidogrel treatment (defined as a maintenance of 75 mg daily therapy for >5 days or a loading dose of 300 mg at least 24h before PCI or 600 mg at least 4 hours before PCI. All patients received aspirin at a dose of 80 to 100 mg daily ≥10days, unless they were on long-term anticoagulation with coumadins. According to our institutional practice, all patients (both after drug eluting and bare-metal stenting) are treated with clopidogrel for at least one year since the year 2003. Clopidogrel and aspirin maintenance doses are 75 mg and 80-100 mg daily respectively. Higher maintenance doses are not used. Compliance to antiplatelet medication is routinely assessed by outpatient visits at 6 weeks, 3 months and 1 year. For patients included in the POPular-study additional telephone contact at 30 days and 12 months were performed. Compliance was also verified by pharmacy refill data.

All interventions were performed according to current guidelines[11] and the choice of stent type and periprocedural use of glycoprotein (GP) IIb/IIIa inhibitors was left to the operator's discretion, but the latter werealways administered after blood collection. Patients using concomitant medication known to affect platelet function other than aspirin (i.e. NSAIDs, dipyramidole, upstream GP IIb/IIIa inhibitors), patients with a known platelet function disorder or a whole blood platelet count <150.000/ μL were excluded. Written informed consent was obtained before PCI. All data were prospectively collected and entered into a central database. Clinical follow-up was obtained by contacting all patients at 30 days and 12 months and a double-check was performed on the basis of source documents obtained from medical records from the referring hospitals.

The study was conducted according to the principles of the Declaration of Helsinki and the

laws and regulations applicable in the Netherlands. The local institutional review board (Verenigde Commissies Mensgebonden Onderzoek [VCMO]) approved the study.

Follow-up and endpoints

The primary endpoint of the POPular-study was defined as a composite of all-cause death, non-fatal myocardial infarction (defined as the occurrence of ischemic symptoms and a spontaneous [i.e. not peri- or post-procedural] troponin T value or creatine kinase MB greater than the upper limit of normal), stent thrombosis (definite stent thrombosis according to the Academic Research Consortion criteria[12]) and ischemic stroke (focal loss of neurologic function caused by an ischemic event). The primary safety endpoint was defined as major or minor bleeding according to the modified Thrombolysis In Myocardial Infarction (TIMI) Study Group criteria.[13]

Exploratory endpoints included elective target vessel revascularization (TVR; revascularization of the vessel treated at the time of inclusion in the study), elective non-target vessel revascularization (non-TVR; revascularization of a vessel different from that treated at the time of enrolment) and hospitalization for ischemia (hospitalization with ischemic symptoms, evidence for ischemia on electrocardiogram, but without elevated cardiac markers).

An independent committee, blinded for platelet function data, adjudicated all endpoints through review of source documents of medical records.

Blood sampling

Before heparinization, whole blood was drawn from the femoral or radial artery sheath. Blood samples were collected into 3.2% citrate tubes for light transmittance aggregometry, (LTA) and the IMPACT-R. The VerifyNow® P2Y12 was performed using Greiner tubes, according to the manufacturer's test protocol. For the PFA-100® System (Siemens Healthcare Diagnostics Products GmbH, Germany) 3.8% buffered citrated blood was used, according to the manufacturer's test protocol. Blood samples for whole blood count were drawn into tubes containing K_3-EDTA and tubes containing PPACK (50 µmol/L) to perform the Plateletworks®.

Platelet Function Measurements

The magnitude of on-treatment platelet reactivity was quantified using the platelet function tests in parallel: LTA with adenosine diphosphate (ADP) 5 and 20 µmol/L as the agonist, the VerifyNow® P2Y12 assay, the Plateletworks® assay using ADP tubes, the IMPACT-*R* assay (both with and without ADP pre-stimulation) and the Dade® PFA Collagen/ADP Test Cartridge (PFA-100® System). Halfway through the POPular-study, the final prototype of the novel INNOVANCE® PFA P2Y* (PFA-100® System) became available for performance evaluation. Except for the INNOVANCE® PFA P2Y*, which is still under development at time of submission, all platelet function tests were commercially available at the start of the study. All platelet function measurements were performed within 2 hours after blood collection.[14, 15, 16, 17, 18]

Platelet Function Tests

Light Transmittance Aggregometry

LTA was quantified in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and maximal (peak) platelet aggregation (%) induced by ADP in final concentrations of 5 and 20 µmol/L was measured.

The VerifyNow® P2Y12 assay

The VerifyNow® system (Accumetrics, San Diego, USA) is a whole blood cartridge-based method to determine the magnitude of ADP-induced platelet agglutination (using 20 µmol/L ADP to induce platelet activation and 22 nmol/L prostaglandin E_1 to decrease the contribution of P2Y1 receptor stimulation by ADP to platelet aggregation.[14,15]

Given the fact that the majority of the studies linking the magnitude of platelet reactivity to the occurrence of atherothrombotic events have used absolute post-clopidogrel platelet reactivity, we preferred using the P2Y12 Reaction Units (PRUs) over the BASE values or % inhibition values, which are also reported by the instrument.

The Plateletworks® assay

The Plateletsworks® assay (Helena Laboratories, Beaumont, Texas) is based on single platelet disappearance. Whole blood samples were collected in tubes containing K_3-EDTA and tubes containing PPACK with 20 µmol/L ADP. A routine platelet count was performed on each sample. The platelet count in the K_3-EDTA tube was used as reference. As the aggregated platelets exceed the threshold limitations for platelet size (<30fL) after stimulation with ADP, they are no longer counted as individual platelets. The ratio between the aggregated platelets in the agonist sample and the platelet count in the reference tube x 100% is used as the degree of platelet aggregation. We recently demonstrated that the Plateletworks® assay is highly time-dependent.[16] Therefore, a cell counter was placed in the catheterization laboratory for rapid measurements between 5 and 10 minutes after blood collection.

The IMPACT-R device

The IMPACT-*R* device (DiaMed, Cresier, Switzerland) is based on the cone and plate(let) analyzer technology.[17] Citrated whole blood samples (130 µL) were placed in a polystyrene well and subjected to a shear rate of $1800s^{-1}$ for 2 minutes using a Teflon Cone. When shear stress is applied, von Willebrand Factor and fibrinogen are instantly immobilized on the polystyrene surface, serving as a substrate for platelet adhesion and subsequent aggregation. The wells were washed and stained with May-Grunwald stain and analyzed with an inverted light microscope connected to an image analysis system. Platelet adhesion and aggregation on the surface were evaluated by examining the percentage of total area covered with platelets designated as surface coverage (SC).

In addition, the IMPACT-*R* ADP was used.[17] With this modified protocol, whole blood samples were pre-stimulated with a sub-maximal concentration ADP (1.38 µM), gently mixed (10 RPM) for 1 minute and then subjected to the IMPACT-R well under defined shear conditions. Exposure to ADP leads to the formation of microaggregates in patients in whom clopidogrel does not effectively inhibit platelet function. These microaggregated platelets temporarily lose their adhesive properties. The percentage SC in the ADP pre-stimulated aliquots is therefore inversely correlated with the magnitude of ADP-induced platelet activation.

PFA-100® System

The PFA-100® System (Siemens Healthcare Diagnostics Products GmbH, Germany),
measures platelet function, in particular adhesion and aggregation, in whole blood under high shear conditions (5000s^{-1}). The time needed to form a platelet plug occluding the aperture cut into a collagen/ADP (COL/ADP)-coated membrane was determined and reported as closure time (CT) in seconds. Furthermore, halfway through the POPular-study a novel PFA-100® test cartridge became available, the final prototype of INNOVANCE® PFA P2Y* (For investigational use only. The performance characteristics of this product have not been established.). The novel test cartridge intents to measure the effect of clopidogrel on platelet function irrespective of the concentration of buffered sodium citrate used for anticoagulation or concurrent therapy with aspirin. Its membrane is coated with 20 µg ADP, 5 ng prostaglandin E1 and 125 µg calcium (as calcium chloride) and the closure time inversely reflects the magnitude of platelet reactivity.[18]

Statistical Analysis

Sample size calculation was based on the ISAR-REACT I trial[19] that included a cohort with similar selection criteria and the same treatment strategy. Therefore, we assumed an incidence of the primary endpoint of 6%. The study was designed on the basis of the superiority principle to have 80 percent power to observe an incidence of the primary end point in patients exhibiting high on-treatment platelet reactivity (HPR) of 10% and 4% in patients without HPR. On this basis, 380 patients were needed in each group. To compensate for loss to follow-up, we aimed for a population of 800 as measured with each test. Owing to irregularities in platelet assay supply as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient. Inclusion continued until at least 4 tests had sufficient power.

Continuous variables are presented as mean ± SD. Categorical data are reported as frequencies (percentages). Categorical variables were compared using the chi-square test. Normally distributed continuous variables were compared with a two-sided unpaired *t* test. Since the PFA-100® System confines detection of a closure time to a 300-s window, and, because the majority of patients on adequate antiplatelet therapy exhibit non-closure according to INNOVANCE® PFA P2Y*, the results of the PFA-100® System are depicted as a Kaplan Meier time-to-aperture-closure plot and a log-rank test was used.

To evaluate a platelet function assay's ability to distinguish between patients with and without primary endpoint at one-year follow-up, a receiver-operator characteristic (ROC) curve analysis was calculated for each test. The optimal cut-off level was calculated by determining the smallest distance between the ROC-curve and the upper left corner of the graph. Patients above the optimal cut-off level were considered to exhibit HPR. Survival analysis for patients with and without HPR according to the ROC of the specific test, were performed using the Kaplan-Meier method, and the differences between groups were assessed by the log-rank test. The measure of effect was the Odds Ratio (OR) and estimated from a logistic regression analysis. A second ROC curve analysis was performed based on the one-year primary safety endpoint; combined TIMI major and minor bleedings.

Logistic regression modelling was used to identify independent correlates of the primary endpoint and to adjust for potential confounders (classic cardiovascular risk factors, renal failure, left ventricular ejection fraction <45%, total stent length, number of lesions treated, amount of stents implanted, bifurcation lesions, co-medication [including use of clopidogrel loading dose, coumadins, proton pump inhibitors, calcium channel blockers, statins or GP IIb/IIIa inhibitors], laboratory parameters [hemoglobin, platelet count and mean platelet volume], left anterior descendens coronary artery (LAD) or graft-stenting). All univariate variables with a p-value <0.10 were included in multivariable analysis. Whether a variable had additional contribution to a logistic regression model without that variable was tested with the likelihood-ratio test. The Hosmer-Le Cessie goodness-of-fit test was performed to assess the adequacy of the model. All statistical analyses were performed with R (version 2.9, http://www.r-project.org) and a two-tailed p-value of <0.05 was considered significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

RESULTS

In total 1328 consecutive patients were invited to participate in the study, 21 (1.6%) refused to participate. Another 238 patients were initially included in the study, but since no stent was implanted they were also excluded (e.g. patients underwent only balloon angioplasty or a fractional flow reserve-measurement demonstrating non-ischemic coronary disease), resulting in a population of 1069 consecutive patients Owing to irregularities in platelet assay supply, particularly in the supply of the Plateletworks®, as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient. As a consequence, LTA was performed in a total of 1049 patients with 5 µmol/L ADP and in 1051 with 20 µmol/L ADP; the VerifyNow® P2Y12 cartridge in 1052 patients; the Plateletworks® assay in 606 patients and the IMPACT-*R* in 910 patients without pre-stimulation and in 905 with ADP-pre-stimulation. The PFA COL/ADP was performed in 812 patients and INNOVANCE® PFA P2Y* in 588 patients.

Baseline characteristics of the cohort are depicted in **Table 1**. Baseline characteristics of the subpopulations according to the tests performed are summarized in **Appendix Table 1**,

Table 1 Baseline characteristics total population

Clinical parameters	Total population
Age	64 ± 10.6
Gender	802/1069
Hypertension	823/1069 (77.0%)
Hypercholesterolemia	858/1069 (80.3%)
Diabetes Mellitus	199/1069 (18.6%)
Family history	646/1069 (60.4%)
Current smoking	119/1069 (11.1%)
LVEF<45%	165/1069 (15.4%)
Renal insufficiency	86/1069 (8.0%)
Prior myocardial infarction	583/1069 (54.5%)
Medication	
Aspirin	955/1068 (89.4%)
Loading dose clopidogrel	548/1068 (51.3%)
PPI	297/1068 (27.8%)
Coumadins	108/1068 (10.1%)
Laboratory Parameters	
Platelet count (x10^9)	271.7 ± 81.6
WBC (x10^9)	7.6 ± 2.3
Hemoglobin (mmol/L)	8.6 ± 2.1
Procedural Parameters	
No.of stents implanted	1669
Minimal stent diameter (mm)	3.1 ± 0.8
Total stent length (mm)	28.1 ± 16.8
Bifurcation lesion	33/1069 (3.1%)
Drug eluting stent	675/1063 (63.5%)
LAD	515 (48.2%)

Table 1: Baseline characteristics of the study population
LVEF = left ventricular ejection fraction; PPI =proton pump inhibitors; CCB = calcium channel blockers; WBC = white bloodcell count, LAD = Left Anterior Descending Artery

Definitions
Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.
Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.
Diabetes mellitus: According to the World Health Organization criteria
Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).
Renal insufficiency: Creatin > 120 µmol/L

demonstrating that the subpopulations tested were well balanced (except for white blood cell counts, p=0.04, all p-values were >0.85)). All patients received optimal clopidogrel pre-treatment; 50.6% received a maintenance dose of 75 mg daily therapy for >5 days, 41.6% received a loading dose of 300 mg at least 24h before PCI and 8.3% received a loading dose of 600 mg at least 4 hours before PCI. One thousand fifty two patients (98.4%) were on 80-100 mg aspirin daily for more than 10 days.

Clinical outcome at 12 months was available for 1067 (99.8%) of the patients. Compliance for clopidogrel was 95.2% after 6 months and 82.1% after one year. During one-year follow-up a total of 18 died (1.7%), 64 (6.0%) patients had non-fatal acute myocardial infarction, 13 (1.2%) presented with definite stent thrombosis and 14 patients suffered from non-fatal ischemic stroke (1.3%). Three possible stent thromboses occurred (0.3%) and no probable stent thromboses were found. A total of 55 (5.1%) patients presented with bleeding; 33 (3.1%) TIMI-major and 24 (2.2%) TIMI-minor bleedings.

Receiver-Operating Characteristic Curve Analysis

Receiver operator characteristic curve (ROC) analysis demonstrated that LTA (both 5 µmol/L ADP and 20 µmol/L), the VerifyNow® P2Y12-cartridge and the Plateletworks® assay were able to distinguish between patients with and without ischemic events at 1-year follow-up. On the contrary, neither the IMPACT-*R* with and without ADP-pre-stimulation, nor the PFA COL/ADP or INNOVANCE® PFA P2Y* were able to discriminate between patients with and without post-procedural events. **Table 2** displays the area under the curve (AUC) and optimal cut-off value for every test **Appendix Figure 1** depicts the optimal cut-off values per test and the percentages of patients exhibiting HPR according to the test. Baseline characteristics for every test, for patients with and without HPR, are depicted in **appendix Table2**, showing significant differences between the two groups.

Logistic regression modelling was used to identify independent predictors for the primary endpoint. The model included on-treatment platelet reactivity according to the various tests as a categorical variable (HPR vs patients without HPR using the cut-off defined with the ROC-analysis) and multiple potential confounders. Independent predictors of 1-year primary endpoint were age (calculated for an increase of 10 years (OR = 1.22; 95%-CI: 0.97-1.51, p=0.08), hypertension (OR = 2.50; 95%-CI: 1.30-4.82, p=0.006), hypercholesterolemia (OR = 0.57; 95%-CI: 0.33-0.98, p=0.04), a left ventricular ejection fraction < 45% (OR = 1.83; 95%-CI: 1.07-3.11, p=0.06) and a prior CABG (OR = 1.91; 95%-CI: 0.96-3.81, p=0.06). Procedural factors independently predicting the primary endpoint were total stent length (OR=0.97, 95%-CI: 0.94-1.00, p=0.05), number of lesions treated (OR = 1.92; 95%-CI: 1.10-3.39, p=0.02), number of stents implanted (OR=2.4, 95%-CI: 1.38-4.30, p=0.002), LAD-stenting (OR = 1.79; 95%-CI: 1.11-2.88, p=0.017) or graft-stenting (OR = 2.88; 95%-CI: 1.00-8.32, p=0.049), stenting a bifurcation lesion (OR =5.43; 95%-CI:1.91-15.45, p=0.002) and a plavix loading dose (OR=1.73, 95%-CI: 2.73-1.09, p=0.02). The remaining variables included for multivariate analysis were not found to be independent correlates of the primary endpoint (p>0.10) and were

Table 2: Area under the curve

AUC and optimal cut-off values for each test									
	AUC	95% CI	Cut-Off	Sensitivity	95% CI	Specificity	95%CI	NPV	PPV
LTA 5 mmol/L	0.63	0.58-0.68	42.9%	60.2	49.8-69.8	59.1	56.0-62.2	94.0%	11.7%
LTA 20 mmol/L	0.62	0.56-0.67	64.5%	54.6	44.2-64.5	63.9	60.8-66.8	93.8%	12.0%
VerifyNow P2Y12	0.62	0.57-0.67	236 PRU	60.4	50.2-69.9	63.1	60.0-66.1	94.3%	13.3%
Plateletworks	0.61	0.53-0.69	80.5%	63.0	49.6-74.6	58.5	54.4-62.6	93.9%	12.6%
IMPACT-*R* spontaneous	0.56	0.50-0.62	SC 8.4	56.4	45.4-66.9	52.5	49.1-55.9	90.0%	7.2%
IMPACT-*R* ADP stimulated	0.53	0.47-0.59	2.0 SC	44.0	33.3-55.3	66.9	63.6-70.0	93.0%	10.7%
PFA-100 COL/ADP	0.50	0.46-0.55	147 seconds	70.0	58.5-79.5	38.4	35.0-42.0	93.1%	9.7%
INNOVANCE® PFA P2Y*	0.56	0.50-0.62	159 seconds	39.1	26.4-53.5	76.2	72.4-79.6	93.7%	12.2%
AUC of different backward regression models for the prediction of the primary end point at one-year follow-up									
			AUC	p-value for addition[c]					
Model 1 : Classic cardiovascular risk factors[a]			0.66						
Model 2: Model 1 + procedural risk factors[b]			0.72	0.0001					
Model 3: Model 2 + HPR									
- LTA peak 5 mmol/L			0.73	0.004					
- LTA peak 20 mmol/L			0.73	0.001					
- VerifyNow P2Y12® cartridge			0.74	0.0002					
- Plateletworks®			0.77	0.001					
- IMPACT-R spontaneous			0.72	0.20					
- IMPACT-R ADP stimulated			0.72	0.13					
- PFA-100 COL/ADP			0.72	0.20					
- INNOVANCE® PFA P2Y*			0.73	0.07					

AUC and optimal cut-off values for each test. AUC = Area under the curve CI = confidence interval NPV = negative predictive value PPV = positive predictive value LTA=light transmittance aggregometry PRU = P2Y12 reaction units SC= surface coverage HPR=high on-treatment platelet reactivity. *AUC of different backward regression models for the prediction of the primary end point at one-year follow-up*

a Age, hypertension, hypercholesterolemia, LVEF (left ventricular ejection fraction) < 45%, prior CABG

b Total stent length, no. of lesions treated, no. of stents implanted, LAD-stenting, graft-stenting, bifurcation lesion, plavix loading dose vs maintenance dose.

c Likelihood Ratio Test for additional value of HPR (increase in AUC) as measured with multiple platelet function tests.

not included in the model.

The addition of HPR to this statistical model revealed that HPR as measured with LTA (both 5 µmol/L ADP and 20 µmol/L), the VerifyNow®-P2Y12-cartridge and the Plateletworks® assay significantly improved the AUC. Likewise, the likelihood-ratio test demonstrated that HPR according to these tests had additional contribution to the model (**Table 3**). The goodness-of-fit test demonstrated that the predicting model was adequate (all p-values>0.10). On the contrary, the AUC did not improve when HPR as measured with IMPACT-*R* (both with and without ADP pre-stimulation) or the PFA Test Cartridges (both PFA COL/ADP and INNOVANCE® PFA P2Y*) was added to the model.

Relationship between high on-treatment platelet reactivity and clinical outcome

At one-year follow-up, the primary endpoint occurred more frequently in patients with HPR compared to patients without HPR when platelet function was evaluated with LTA (11.7% vs 6.0%, p=0.0009 using 5 µmol/L ADP and 12.0% vs 6.2%, p=0.001 using 20 µmol/L ADP respectively), the VerifyNow® P2Y12 assay (13.3% vs 5.7%, p<0.0001), the Plateletworks® assay (12.6% vs 6.1%, p=0.005) and the INNOVANCE® PFA P2Y* (12.2% vs. 6.3%, p=0.02). One-year follow-up for patients with and without HPR according to each platelet function test is depicted in **Table 3**.

The survival rate free from the primary endpoint was significantly lower in patients with HPR when measured with LTA 5 µmol/L ADP, LTA 20 µmol/L ADP, VerifyNow®, Plateletworks® and INNOVANCE® PFA P2Y* as compared to patients without HPR, whereas no significant relation was detected when platelet function was assessed by the IMPACT-*R* (both with and without pre-stimulation) or by the PFA COL/ADP (**Figure 1**).

The occurrence of the primary end point was also compared when groups were divided in quintiles according to on-treatment platelet reactivity (**Figure 2**). Patients in the higher quintiles according to the LTA 5 µmol/L ADP and 20 µmol/L ADP and the VerifyNow® P2Y12 assay were at significantly higher risk for the primary end point. In contrast, no significant difference in the occurrence of the primary endpoint was observed between quintiles as measured with the IMPACT-*R* tests and Plateletworks®. Since the PFA-100® System confines detection of a closure time to a 300-s window, the results of both PFA-cartridges are depicted as time to aperture closure Kaplan-Meier curves. Closure times as measured by the PFA COL/ADP were not significantly different between patients with and without a primary endpoint.

Relationship between platelet reactivity and bleeding

A second ROC-analysis demonstrated that none of the performed tests was able to discriminate between patients with and without bleeding (all AUC's included 0.50 in the confidence interval [CI])). Stratification by quintiles based on on-treatment platelet reactivity demonstrated no significant difference in the occurrence of bleeding between the quintiles (**Appendix Figure 2**). In addition, no significant increase in bleeding was observed in the lowest quintile of patients compared to

Table 3: Clinical Outcome

	5 µmol/L ADP NPR (n=604) < 42.9 % aggregation	HPR (n=445) > 42.9 % aggregation	OR (95 CI)	p-value
Death, MI, ST, stroke	36 (6.0%)	52 (11.7%)	2.09 (1.34 – 3.25)	0.0009
Death	6 (1.0%)	11 (2.5%)	2.53 (0.93 - 6.88)	0.06
MI	24 (4.0%)	37 (8.3%)	2.19 (1.29 - 3.72)	0.003
ST	6 (1.0%)	7 (1.6%)	1.59 (0.53 – 4.77)	0.40
Stroke	7 (1.2%)	6 (1.3%)	1.17 (0.39 – 3.49)	0.78
TVR	18 (3.0%)	7 (1.6%)	0.52 (0.22 – 1.26)	0.14
Non-TVR	21 (3.5%)	8 (1.8%)	0.51 (0.22-1.16)	0.10
Rehospitalization	16 (2.6%)	11 (2.5%)	0.93 (0.43 – 2.03)	0.87

	20 µmol/L ADP NPR (n=659) < 64.5 % aggregation	HPR (n=392) > 64.5 % aggregation	OR (95 CI)	p-value
Death, MI, ST, stroke	41 (6.2%)	47 (12.0%)	2.05 (1.32 - 3.19)	0.001
Death	11 (1.7%)	6 (1.5%)	0.92 (0.34 - 2.50)	0.86
MI	24 (3.6%)	37 (9.4%)	2.76 (1.62 – 4.68)	0.0001
ST	4 (0.6%)	9 (2.3%)	3.85 (1.18 – 12.58)	0.017
Stroke	8 (1.2%)	5 (1.3%)	1.05 (0.34 – 3.24)	0.93
TVR	21 (3.2%)	4 (1.0%)	0.31 (0.11 – 0.92)	0.03
Non-TVR	23 (3.5%)	6 (1.5%)	0.43 (0.17 – 1.07)	0.06
Rehospitalization	21 (3.2%)	6 (1.5%)	0.47 (0.19 – 1.18)	0.10

	VerifyNow P2Y12 NPR (n=646) < 236 PRU	HPR (n=406) > 236 PRU	OR (95% CI)	p-value
Death, MI, ST, stroke	37 (5.7%)	54 (13.3%)	2.53 (1.63 – 3.91)	<0.0001
Death	9 (1.4%)	9 (2.2%)	1.60 (0.63 - 4.08)	0.32
MI	23 (3.6%)	40 (9.9%)	2.96 (1.74 – 5.02)	<0.0001
ST	5 (0.8%)	8 (2.0%)	2.58 (0.84 - 7.93)	0.09
Stroke	6 (0.9%)	7 (1.7%)	1.87 (0.62 – 5.61)	0.26
TVR	16 (2.5%)	9 (2.2%)	0.89 (0.39 - 2.04)	0.79
Non-TVR	20 (3.1%)	9 (2.2%)	0.71 (0.32 - 1.57)	0.40
Rehospitalization	18 (2.8%)	8 (2.0%)	0.70 (0.30 - 1.63)	0.41

	Plateletworks[ò] NPR (n=344) < 80.5 %aggregation	HPR (n=262) > 80.5 %aggregation	OR (95% CI)	p-value
Death, MI, ST, stroke	21 (6.1%)	33 (12.6%)	2.22 (1.25 – 3.93)	0.005
Death	9 (2.6%)	4 (1.5%)	0.58 (0.18 - 1.89)	0.36
MI	10 (2.9%)	25 (9.5%)	3.52 (1.66 - 7.47)	0.0005
ST	3 (0.9%)	6 (2.3%)	2.66 (0.66 - 10.75)	0.15
Stroke	3 (0.9%)	4 (1.5%)	1.76 (0.39 - 7.94)	0.45
TVR	12 (3.5%)	5 (1.9%)	0.54 (0.19 - 1.55)	0.24
Non-TVR	11 (3.2%)	7 (2.7%)	0.83 (0.32 - 2.17)	0.71
Rehospitalization	10 (2.9%)	7 (2.7%)	0.92 (0.34 - 2.44)	0.86

	IMPACT-R NPR (n=481) SC < 8.4	HPR (n=429) SC > 8.4	OR (95% CI)	p-value
Death, MI, ST, stroke	36 (7.5%)	42 (9.8%)	1.34 (0.84 – 2.14)	0.21
Death	5 (1.0%)	11 (2.6%)	2.51 (0.86 – 7.27)	0.08
MI	28 (5.8%)	25 (5.8%)	1.00 (0.57 - 1.75)	0.99
ST	5 (1.0%)	6 (1.4%)	1.35 (0.41 – 4.46)	0.62
Stroke	4 (0.8%)	7 (1.6%)	1.98 (0.58 – 6.8)	0.27
TVR	15 (3.1%)	6 (1.4%)	0.44 (0.17 – 1.15)	0.08
Non-TVR	15 (3.1%)	9 (2.1%)	0.67 (0.29 – 1.54)	0.33
Rehospitalization	12 (2.5%)	12 (2.8%)	1.12 (0.5 – 2.53)	0.78

	IMPACT-R ADP NPR (n=609) SC > 2.0	HPR (n=296) SC ≤ 2.0	OR (95% CI)	p-value
Death, MI, ST, stroke	43 (7.1%)	32 (10.8%)	1.60 (0.99-2.58)	0.05
Death	9 (1.5%)	6 (2.0%)	1.38 (0.49-3.91)	0.54
MI	29 (4.8%)	22 (7.4%)	1.61 (0.91-2.85)	0.10
ST	7 (1.1%)	3 (1.0%)	0.88 (0.23-3.43)	0.85
Stroke	7 (1.1%)	4 (1.4%)	1.18 (0.34-4.06)	0.79
TVR	12 (2.0%)	9 (3.0%)	1.56 (0.65-3.75)	0.32
Non-TVR	15 (2.5%)	9 (3.0%)	1.24 (0.54-2.87)	0.61
Rehospitalization	17 (2.8%)	7 (2.4%)	0.84 (0.35-2.06)	0.71

	PFA 100 COL/ADP NPR (n=306) CT>147	HPR (n=506) CT≤ 147	OR (95% CI)	p-value
Death, MI, ST, stroke	21/306 (6.9%)	49/506 (9.7%)	1.46 (0.85-2.48)	0.17
Death	5/306 (1.6%)	10/506 (2.0%)	1.21 (0.41-3.58)	0.73
MI	16/306 (5.2%)	4/506 (6.7%)	1.31 (0.71-2.41)	0.39
ST	4/306 (1.3%)	5/506 (1.0%)	0.75 (0.20-2.83)	0.67
Stroke	1/306 (0.3%)	7/506 (1.4%)	4.28 (0.52-34.90)	0.14
TVR	11/306 (3.6%)	9/506 (1.8%)	0.49 (0.20-1.19)	0.11
Non-TVR	11/306 (3.6%)	12/506 (2.4%)	0.65 (0.28-1.50)	0.31
Rehospitalization	9/306 (2.9%)	7/506 (1.4%)	0.46 (0.17-1.26)	0.12

	INNOVANCE® PFA P2Y* NPR (n=441) CT>159	HPR (n=147) CT≤ 159	OR (95% CI)	p-value
Death, MI, ST, stroke	28/441 (6.3%)	18/147 (12.2%)	2.06 (1.10-3.84)	0.02
Death	4/441 (0.9%)	6/147 (4.1%)	4.65 (1.29-16.70)	0.01
MI	20/441 (4.5%)	11/147 (7.5%)	1.70 (0.80-3.64)	0.17
ST	4/441 (0.9%)	1/147 (0.7%)	0.75 (0.08-6.75)	0.80
Stroke	5/441 (1.1%)	1/147 (0.7%)	0.60 (0.07-5.15)	0.65
TVR	16/441 (3.6%)	1/147 (0.7%)	0.18 (0.02-1.38)	0.06
Non-TVR	14/441 (3.2%)	1/147 (0.7%)	0.21 (0.03-1.60)	0.10
Rehospitalization	12/441 (2.7%)	1/147 (0.7%)	0.24 (0.03-1.90)	0.15

HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity, MI = myocardial infarction, ST = Stent thrombosis, TVR = target-vessel revascularization, non-TVR=non-target vessel revascularization.

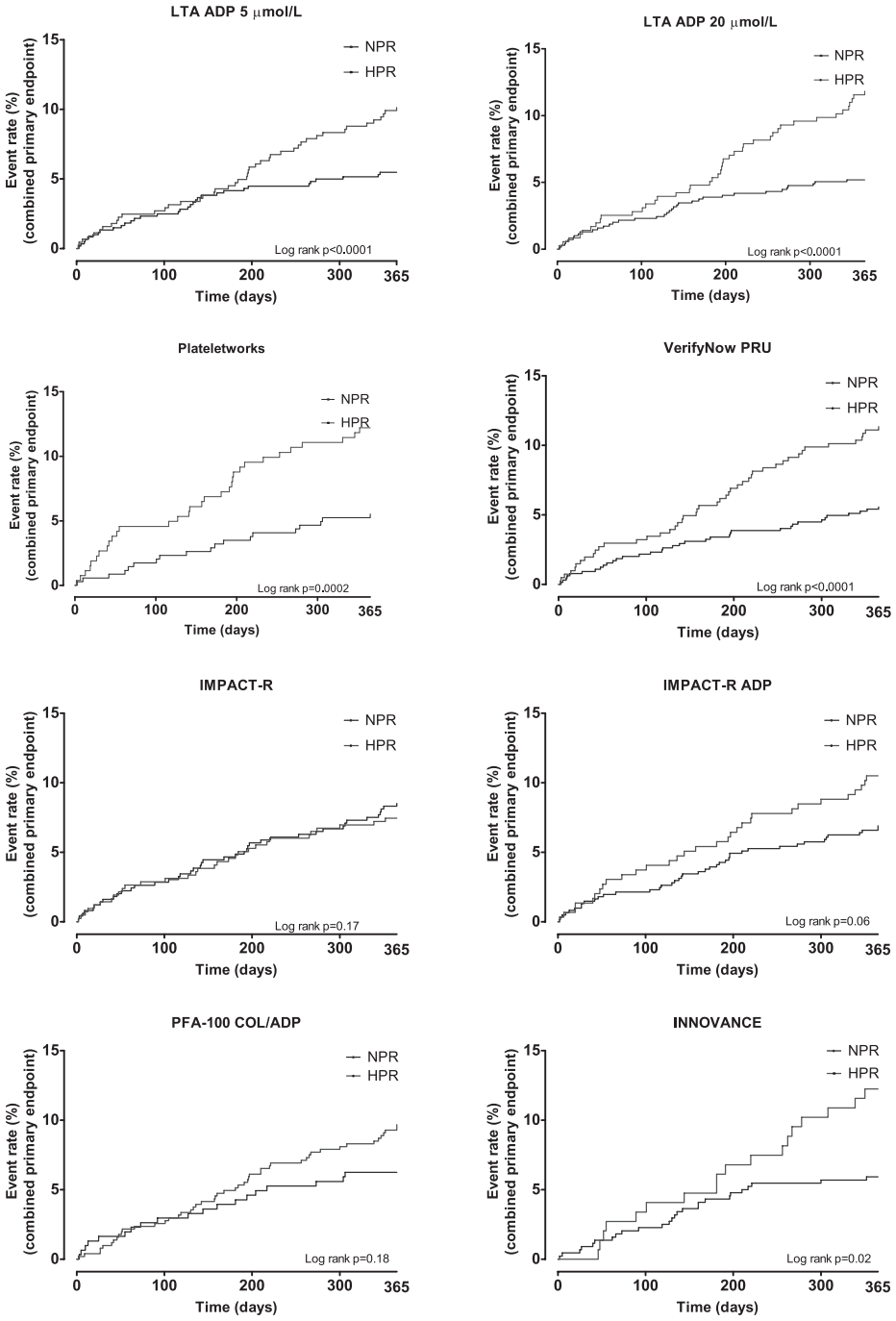


Figure 1: Kaplan-Meier Analysis

Kaplan Meier analysis for the survival free from the combined primary endpoint in patients with and without HPR as measured by the multiple platelet function assays.

HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity

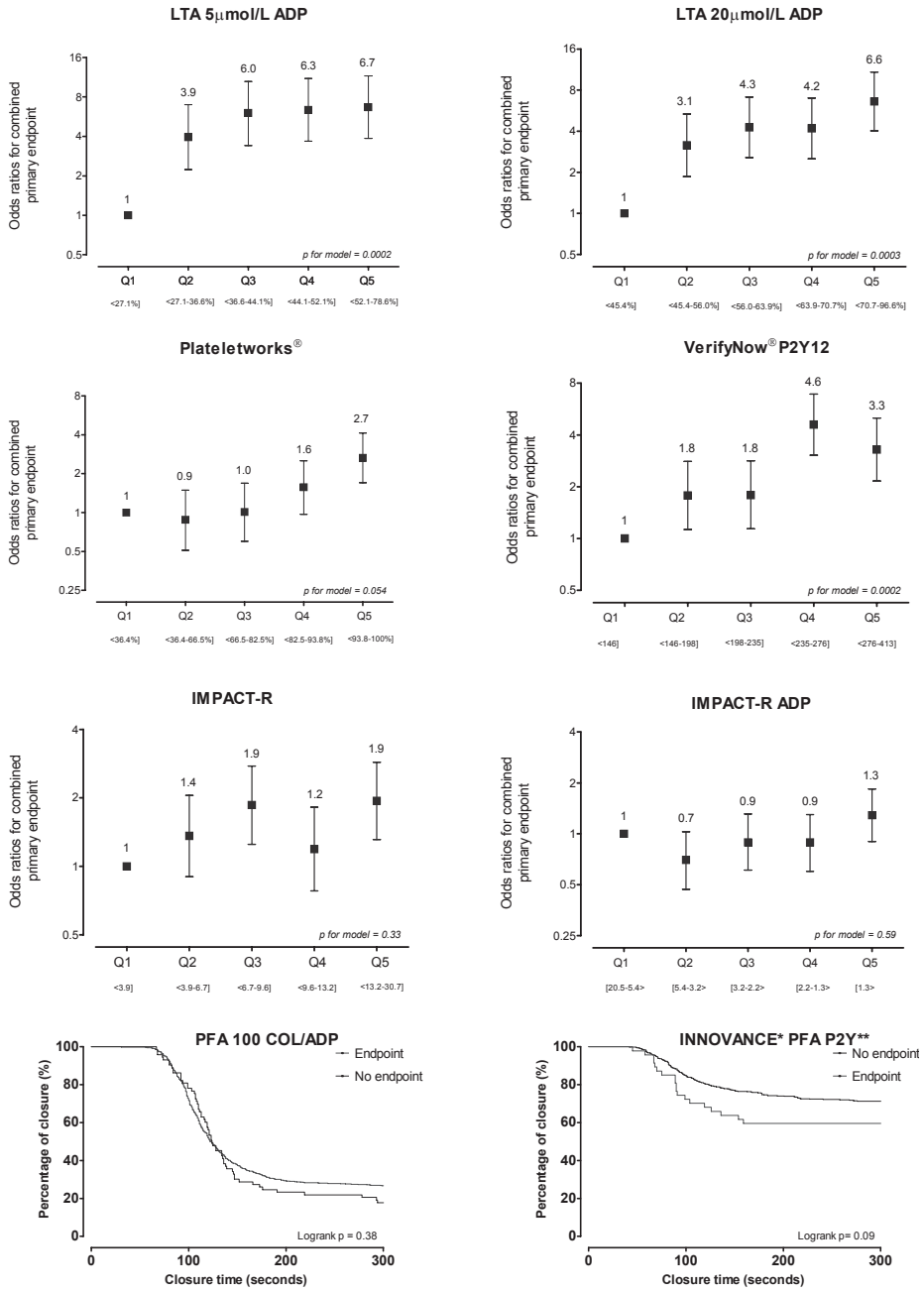


Figure 2: Odds Ratios for the primary endpoint

Odds Ratios for the combined primary endpoint by quintiles of on-treatment platelet reactivity according to the multiple platelet function assays. Incidences (%) of the combined primary endpoint are depicted in the bars. Cumulative Kaplan Meier time-to-aperture-closure plot in patients with and without the combined primary endpoint according to the PFA-100® System.

Q = quintile HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity

quintiles 2 to 5. A third ROC-analysis further demonstrated that the platelet function tests were not able to predict post-discharge (>48 hrs) minor or major bleedings (all AUC's included 0.50 in the confidence interval [CI])

DISCUSSION

The POPular-study is a prospective study evaluating the capability of multiple platelet function to predict clinical outcome in clopidogrel-treated patients undergoing elective PCI with stent implantation.

High on-treatment platelet reactivity when assessed by LTA (both 5 µmol/L and 20 µmol/L ADP) and the VerifyNow® P2Y12 assay, the Plateletworks® and INNOVANCE® PFA P2Y* is significantly associated with atherothrombotic events. In contrast, the shear stress based tests IMPACT-*R* (both with and without ADP pre-stimulation) and the Dade® PFA-100 COL/ADP-cartridge did not show an association with outcome.

The 'gold standard' LTA has been the most widely used technique and has clearly demonstrated the relationship between high-on treatment platelet reactivity and subsequent atherothrombotic events.[4-6] The POPular-study found an optimal diagnostic cut-off level discriminating patients with atherothrombotic events from those who were uneventful similar to that found by Gurbel et al. However, LTA is not suitable for routine use in clinical practice due to the poor reproducibility, the long sample processing time and the need for specialized technicians. Therefore, several new more easy to use platelet function tests have been introduced. The POPular-study reveals that the VerifyNow® P2Y12 cartridge is capable of identifying patients who are at risk for atherothrombotic events post-PCI. Our optimal diagnostic cut-off value of 236 PRU is perfectly consistent with that reported in previous reports.[7,8,20] The POPular-study is the largest study to demonstrate a relation between the Plateletworks® ADP assay and clinical outcome and the first to establish an optimal cut-off value. The results seem promising with the largest increase in predictive value of all tests performed in the POPular-study. However, rapid performance (within 10 minutes after blood withdrawal) of this assay is required, since the ADP-induced platelet aggregates disaggregate after this time-point, resulting in a unreliable test result as descibed in the Online Supplement.[16] Therefore, the use of the Plateletworks® in routine clinical practice might be limited.

The POPular-study also reports performance data of the prototype INNOVANCE® PFA P2Y*, which in its final design became available halfway through the inclusion-period. A lower incidence of the primary endpoint in patients without high on-treatment platelet reactivity was demonstrated. However, high on-treatment platelet reactivity as measured with INNOVANCE® PFA P2Y* did not improve the predictability of the risk-model.

In the light of the POPular data, should high on-treatment platelet reactivity be used as a prognostic marker in clinical practice? Despite growing evidence that high on-treatment platelet reactivity is associated with adverse clinical outcome, platelet function testing is not widely implemented in clinical practice due to a lack of consensus on the optimal method and on the

optimal cut-off values of the different tests to identify patients at higher risk. The POPular-study provides additional evidence - including optimal cut-off values - that three tests might be used (LTA, VerifyNow® and Plateletworks®). Although the sample size has insufficient statistical power, the novel INNOVANCE® PFA P2Y* seems promising for this purpose as well. However, also other risk factors such as diabetes mellitus and poor left ventricular function have been demonstrated to predict atherothrombotic events post-stent implantation.[21,22,23,24] Furthermore, these same risk factors have been shown to be associated with high on-treatment platelet reactivity[25,26] and thus, high on-treatment platelet reactivity is probably a composite of several of these risk factors as well as the response to antiplatelet therapy.

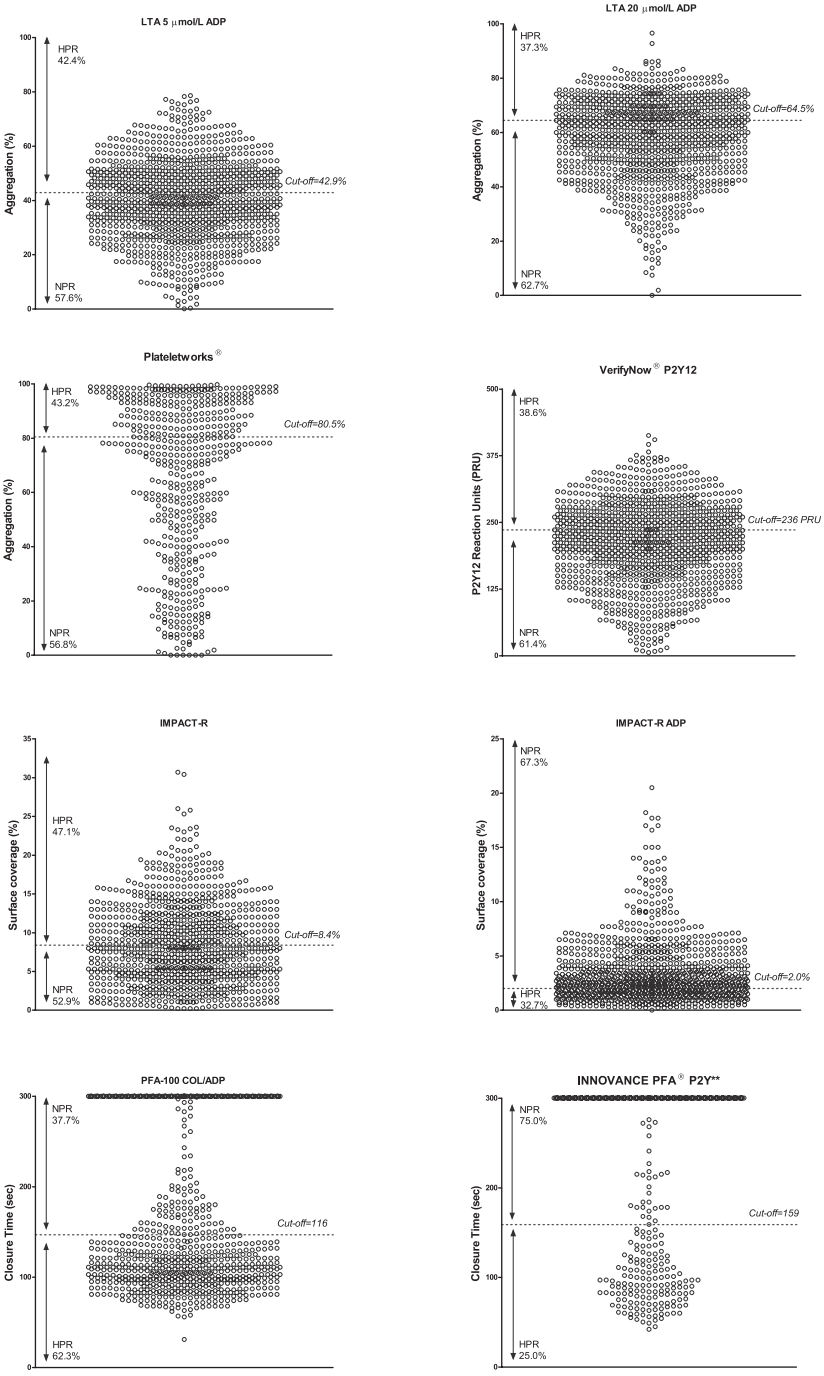
In the POPular-study high on-treatment platelet reactivity indeed added to the overall risk-model. The modest contribution of high on-treatment platelet reactivity in the POPular-study might be attributed to its relatively low risk population, excluding higher-risk patients (in particular ST-elevation myocardial infarction). The greater importance of high on-treatment platelet reactivity in patients at higher risk, has been demonstrated by Sibbing and colleagues and Marcucci and colleagues.[7,9]

Despite numerous data on the association between high on-treatment platelet reactivity and adverse outcome there is only preliminary data concerning the benefit of tailoring therapy based on the results of platelet function testing.[27] Therefore, the correct treatment-if any-of high on-treatment platelet reactivity remains unknown and we have to await currently ongoing clinical trials; the GRAVITAS (NCT00645918), the DANTE (NCT00774475), the ARCTIC (NCT00827411) - randomizing patients to higher clopidogrel doses versus routine doses based on platelet function testing as well as the TRIGGER-PCI (NCT00910299) randomizing to prasugrel versus clopidogrel - which will reveal whether individualized antiplatelet treatment based on platelet function testing improves outcome. Until than clinical practice should not be guided by (point-of-care) platelet function testing.

Some issues merit careful consideration. First, the sample size of INNOVANCE® PFA P2Y* was too small to have sufficient statistical power to detect the relationship between high on-treatment platelet reactivity and clinical outcome . Second, not all currently available platelet function tests were included. Additional tests include the Multiplate, the thromboelastograph and the Flowcytometric Vasodilator-Stimulated Phosphoprotein (VASP)-analysis. However, at the start of our inclusion the Multiplate and the platelet assay for the thromboelastograph were not available. Furthermore, the published results with the VASP-assay were mainly preliminary and did not provide a solid base for choosing VASP as one of the platelet function tests. Third, patients received three different, but adequate, clopidogrel dosing strategies. Previous studies have demonstrated differences in the effect on platelet reactivity of these three dosing regimes. However, these three regimens are current clinical practice, and the POPular-study therefore reflects the clinical relevance of monitoring platelet function in daily practice.

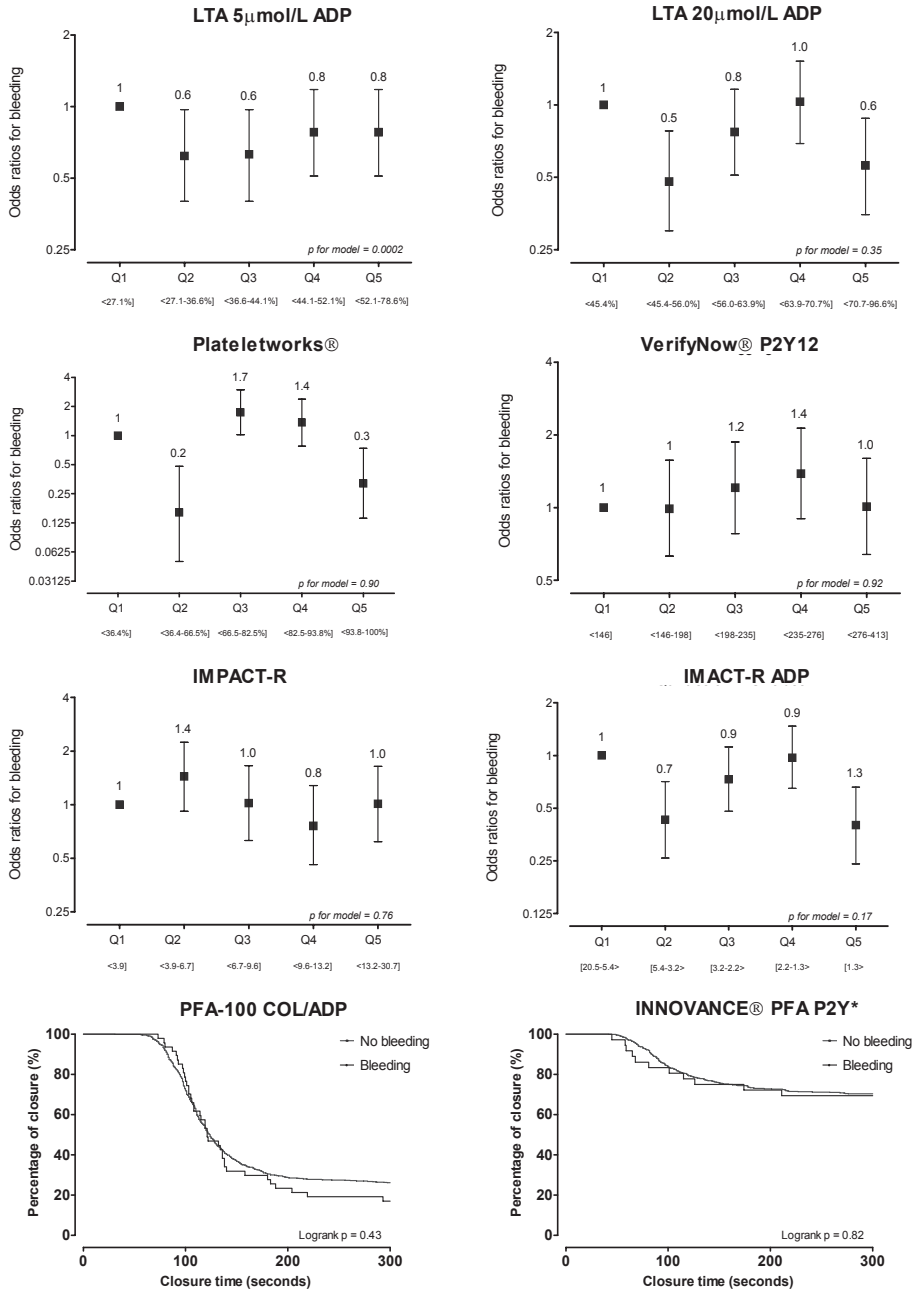
In conclusion, of the platelet function tests assessed, only LTA, VerifyNow®, Plateletworks® and INNOVANCE® PFA P2Y* were significantly associated with the primary endpoint. However, the

predictability of these four tests was only modest. None of the tests provided accurate prognostic information to identify patient at higher risk of bleeding. Thus, the POPular-study does not support the use of platelet function testing to guide clinical practice in a low-risk population of patients undergoing elective PCI.



Appendix Figure 1: Distribution of individual platelet reactivity

Individual platelet reactivity data obtained from the multiple platelet function assays.
Horizontal dotted lines indicate the test specific cut-off values for high-on treatment platelet reactivity
HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity



Appendix Figure 2: Odds Ratios for the primary safety endpoint

Odds Ratios for the occurrence of bleeding by quintiles of on-treatment platelet reactivity according to the multiple platelet function assays. Incidences (%) of the combined primary endpoint are depicted in the bars. Cumulative Kaplan Meier time-to-aperture-closure plot in patients with and without bleeding according to the PFA-100® System.

Q = quintile HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity

Appendix Table 1

Clinical parameters	LTA 5 µmol/L ADP n=1049	LTA 20 µmol/L ADP n=1051	VerifyNow P2Y12 n=1052	Plateletworks n=606
Age (yrs)	64 ± 10.6	64 ± 10.6	64 ± 10.6	64 ± 10.6
BMI (kg/m2)	27.2 ± 4.0	27.2 ± 4.0	27.2 ± 4.0	27.2 ± 3.8
Gender (m/f)	784/265	786/265	790/262	458/148
Hypertension	810/1049 (77.2%)	812/1051 (77.3%)	812/1052 (77.2%)	461/606 (76.1%)
Hypercholesterolemia	841/1049 (80.2%)	843/1051 (80.2%)	843/1052 (80.1%)	479/606 (79.0%)
Diabetes Mellitus	194/1049 (18.5%)	195/1051 (18.6%)	194/1052 (18.4%)	109/606 (18.0%)
Family History	637/1049 (60.7%)	638/1051 (60.7%)	634/1052 (60.3%)	365/606 (60.2%)
Current smoking	115/1049 (11.0%)	116/1051 (11.0%)	116/1052 (11.0%)	60/606 (9.9%)
LVEF < 45%	161/1049 (15.3%)	160/1051 (15.2%)	163/1052 (15.5%)	96/606 (15.8%)
Renal insufficiency	85/1049 (8.1%)	85/1051 (8.1%)	82/1052 (7.8%)	46/606 (7.6%)
Prior myocardial infarction	576/1049 (54.9%)	577/1051 (54.9%)	573/1052 (54.5%)	320/606 (52.8%)
Prior PCI	341/1049 (32.5%)	340/1051 (32.4%)	340/1052 (32.3%)	204/606 (33.7%)
Prior CABG	110/1049 (10.5%)	111/1051 (10.6%)	107/1052 (10.2%)	67/606 (11.1%)
Medication				
Aspirin	936/1048 (89.2%)	938/1050 (89.2%)	940/1051 (89.4%)	544/605 (89.8%)
Loading dose clopidogrel	533/1048 (50.9%)	534/1050 (50.9%)	536/1051 (51.0%)	305/605 (50.4%)
Statin	833/1048 (79.5%)	834/1050 (79.4%)	834/1051 (79.4%)	467/605 (77.2%)
Beta-blocker	799/1048 (76.2%)	800/1050 (76.2%)	802/1051 (76.3%)	463/605 (76.5%)
ACE-inhibitor	392/1048 (37.4%)	392/1050 (37.3%)	391/1051 (37.2%)	220/605 (36.4%)
PPI	296/1048 (28.2%)	296/1050 (28.2%)	295/1051 (28.1%)	159/605 (26.3%)
CCB	394/1048 (37.6%)	394/1050 (37.5%)	397/1051 (37.8%)	245/605 (40.5%)
Oral antidiabetics	70/1048 (6.6%)	71/1050 (6.8%)	69/1051 (6.6%)	40/605 (6.6%)
Coumadins	108/1048 (10.3%)	108/1050 (10.3%)	106/1051 (10.1%)	48/605 (7.9%)
Laboratory Parameters				
Platelet count (x10^9)	271.1 ± 79.3	271.1 ± 79.3	271.8 ± 81.7	274.5 ± 82.7
WBC (x10^9)	7.9 ± 8.5	7.9 ± 8.5	7.9 ± 8.5	8.1 ± 10.6
Hemoglobin (mmol/L)	8.6 ± 2.1	8.6 ± 2.1	8.6 ± 2.1	8.7 ± 2.6
Procedural Parameters				
No.of stents implanted	1635	1656	1646	936
No.of lesions treated	1448	1452	1454	833
Minimal Stent diameter (mm)	3.1 ± 0.8	3.1 ± 0.8	3.1 ± 0.8	3.1 ± 0.5
Total Stent length (mm)	28.1 ± 16.9	28.2 ± 16.9	28.1 ± 16.8	28.2 ± 16.5
Bifurcation lesion	31/1049 (3.0%)	32/1050 (3.0%)	33//1052 (3.1%)	15/606 (2.5%)
Drug eluting stent	594/1043 (57.0%)	596/1045 (57.0%)	598/1047 (57.1%)	358/602 (59.5%)
LAD	506/1049 (48.2%)	505/1050 (48.0%)	514/1052 (48.9%)	300/606 (49.5%)
Graft	33/1049 (3.1%)	33/1050 (3.1%)	31/1052 (2.9%)	16/606 (2.6%)

Events				
Death, MI, ST, stroke	88/1049 (8.4%)	88/1051 (8.4%)	91/1052 (8.7%)	54/606 (8.9%)
Death	17/1049 (1.6%)	17/1051 (1.6%)	18/1052 (1.7%)	13/606 (2.1%)
MI	61/1049 (5.8%)	61/1051 (5.8%)	63/1052 (6.0%)	35/606 (5.7%)
ST	13/1049 (1.2%)	13/1051 (1.2%)	13/1052 (1.2%)	9/606 (1.2%)
Stroke	13/1049 (1.2%)	13/1051 (1.2%)	13/1052 (1.2%)	7/606 (1.2%)
TVR	25/1049 (2.4%)	25/1051 (2.4%)	25/1052 (2.4%)	17/606 (2.8%)
Non-TVR	29/1049 (2.8%)	29/1051 (2.8%)	29/1052 (2.8%)	18/606 (3.0%)
Rehospitalization	27/1049 (2.6%)	27/1051 (2.6%)	26/1052 (2.5%)	17/606 (2.8%)

Clinical parameters	IMPACT-*R* n=910	IMPACT-*R* ADP n=905	PFA-100 COL/ADP n=812	INNOVANCE® PFA P2Y* n=588
Age (yrs)	64 ± 10.7	64 ± 10.7	64 ± 10.5	65 ± 10.7
BMI (kg/m2)	27.3 ± 4.1	27.3 ± 4.1	27.2 ± 4.1	27.2 ± 4.3
Gender (m/f)	683/227	680/225	597/215	424/164
Hypertension	708/910 (77.8%)	704/905 (77.8%)	620/812 (76.4%)	436/588 (74.1%)
Hypercholesterolemia	732/910 (80.4%)	724/905 (80.4%)	652/812 (80.3%)	464/588 (78.9%)
Diabetes Mellitus	166/910 (18.2%)	165/905 (18.2%)	150/812 (18.5%)	113/588 (19.2%)
Family History	544/910 (59.8%)	539/905 (59.6%)	488/812 (60.1%)	344/588 (58.5%)
Current smoking	98/910 (10.8%)	95/905 (10.5%)	84/812 (10.3%)	61/588 (10.4%)
LVEF < 45%	132/910 (14.5%)	133/905 (14.7%)	117/812 (14.4%)	91/588 (15.5%)
Renal insufficiency	72/910 (7.9%)	72/905 (8.0%)	58/812 (7.1%)	48/588 (8.2%)
Prior MI	500/910 (54.9%)	497/905 (54.9%)	462/812 (56.9%)	339/588 (57.7%)
Prior PCI	292/910 (32.1%)	290/905 (32.0%)	246/812 (30.3%)	171/588 (29.1%)
Prior CABG	100/910 (11.0%)	100/905 (11.0%)	91/812 (11.2%)	67/588 (11.4%)
Medication				
Aspirin	807/909 (88.7%)	801/904 (88.5%)	716/811 (88.3%)	513/588 (87.2%)
Loading dose clopidogrel	464/909 (51.0%)	463/904 (51.2%)	395/811 (48.7%)	288/588 (49.0%)
Statin	732/909 (80.5%)	729/904 (80.6%)	643/811 (79.3%)	457/588 (77.7%)
Beta-blocker	701/909 (77.1%)	695/904 (76.9%)	622/811 (76.7%)	453/588 (77.0%)
ACE-inhibitor	334/909 (36.7%)	332/904 (36.7%)	310/811 (38.2%)	224/588 (38.1%)
PPI	241/909 (26.5%)	240/904 (26.5%)	229/811 (28.2%)	181/588 (30.8%)
CCB	344/909 (37.8%)	344/904 (38.1%)	303/811 (37.4%)	212/588 (36.1%)
Oral antidiabetics	56/909 (6.2%)	55/904 (6.1%)	53/811 (6.5%)	36/588 (6.1%)
Coumadins	90/909 (9.9%)	91/904 (10.1%)	87/811 (10.7%)	73/588 (12.4%)

Laboratory Parameters				
Platelet count (x10^9)	271.9 ± 81.8	271.9 ± 81.6	271.3 ± 82.3	264.5 ± 76.5
WBC (x10^9)	8.0 ± 8.9	8.0 ± 8.9	8.0 ± 9.4	8.1 ± 11.0
Hemoglobin (mmol/L)	8.6 ± 2.2	8.5 ± 2.2	8.6 ± 2.3	8.6 ± 2.7
Procedural Parameters				
No.of stents implanted	1421	1406	1255	893
No.of lesions treated	1253	1243	1098	775
Minimal Stent diameter (mm)	3.1 ± 0.86	3.1 ± 0.86	3.1 ± 0.8	3.1 ± 1.0
Total Stent length (mm)	28.1 ± 16.6	27.9 ± 16.4	27.6 ± 16.3	26.7 ± 15.4
Bifurcation lesion	25/910 (2.7%)	25/605 (2.8%)	20/812 (2.5%)	12/588 (2.0%)
Drug eluting stent	515/905 (56.9%)	513/900 (57%)	445/810 (54.9%)	328/588 (55.8%)
LAD	437/910 (48.0%)	435/905 (48.1%)	399/812 (49.1%)	291/588 (49.5%)
Events				
Death, MI, ST, stroke	78/910 (8.6%)	65/905 (7.2%)	70/812 (8.6%)	46/588 (7.8%)
Death	16/910 (1.8%)	15/905 (1.7%)	15/812 (1.8%)	10/588 (1.7%)
MI	53/910 (5.8%)	51/905 (5.6%)	50/812 (6.2%)	31/588 (5.3%)
ST	11/910 (1.2%)	10/905 (1.1%)	9/812 (1.1%)	5/588 (0.9%)
Stroke	11/910 (1.2%)	11/905 (1.2%)	8/812 (1.0%)	6/588 (1.0%)
TVR	21/910 (2.3%)	21/905 (2.3%)	21/812 (2.6%)	17/588 (2.9%)
Non-TVR	24/910 (2.6%)	24/905 (2.7%)	23/812 (2.8%)	15/588 (2.6%)
Rehospitalization	24/910 (2.6%)	24/905 (2.7%)	16/812 (2.0%)	13/588 (2.2%)
Bleeding	47/910 (5.2%)	48/905 (5.3%)	47/812 (5.8%)	36/588 (6.1%)
CAB-related bleeding	6/910 (0.7%)	6/905 (0.7%)	8/812 (1.0%)	6/588 (1.0%)

Appendix Table 1: Baseline characteristics of the subpopulations according to the available platelet function measurements

BMI = Body Mass Index; LVEF = left ventricular ejection fraction; PCI = percutaneous coronary intervention; CABG = coronary artery bypass grafting; PPI =proton pump inhibitors CCB = calcium channel blockers; WBC = white bloodcell count, LAD = Left Anterior Descending Artery; MI=myocardial infarction; ST=stent thrombosis; TVR= target vessel Revascularization; non-TVR=non-target vessel revascularization

Definitions as in Table 1:

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.

Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.

Diabetes mellitus: According to the World Health Organization criteria

Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).

Renal insufficiency: Creatin > 120 µmol/L

Appendix Table 2: Baseline characteristics of the study population according to the magnitude of platelet reactivity

	LTA 5 µmol/L ADP			LTA 20 µmol/L ADP		
Clinical parameters	NPR (n= 604)	HPR (n=445)	p-value	NPR (n=659)	HPR (n=392)	p-value
Age (yrs)	63 ± 10.7	66 ± 10.2	<0.0001	63 ± 10.5	65 ± 10.7	0.002
BMI (kg/m2)	26.9 ± 4.0	27.6 ± 4.0	0.006	26.8 ± 3.9	27.9 ± 4.1	<0.0001
Gender (m/f)	463/141	321/124	0.10	506/153	280/112	0.53
Hypertension	458/604 (75.8%)	352/445 (79.1%)	0.21	503/659 (76.3%)	309/392 (78.8%)	0.05
Hypercholesterolemia	490/604 (81.1%)	351/445 (78.9%)	0.37	534/659 (81.0%)	309/392 (78.8%)	0.39
Diabetes Mellitus	88/604 (14.6%)	106/445 (23.8%)	0.0001	102/659 (15.5%)	93/392 (23.7%)	0.0009
Family History	357/604 (59.1%)	280/445 (62.9%)	0.21	388/659 (58.9%)	250/392 (63.8%)	0.12
Current smoking	74/604 (12.3%)	41/445 (9.2%)	0.12	78/659 (11.8%)	38/392 (9.7%)	0.28
LVEF < 45%	84/604 (13.9%)	77/445 (17.3%)	0.13	89/659 (13.5%)	71/392 (18.1%)	0.04
Renal insufficiency	38/604 (6.3%)	47/445 (10.6%)	0.01	54/659 (8.2%)	31/392 (7.9%)	0.87
Prior MI	338/604 (56.0%)	238/445 (53.5%)	0.43	354/659 (53.7%)	223/392 (56.9%)	0.32
Prior PCI	196/604 (32.5%)	145/445 (32.6%)	0.96	209/659 (31.7%)	131/392 (33.4%)	0.57
Prior CABG	60/604 (9.9%)	50/445 (11.2%)	0.50	70/659 (10.6%)	41/392 (10.5%)	0.93
Medication						
Aspirin	545/604 (90.2%)	391/444 (88.1%)	0.26	597/659 (90.6%)	341/391 (87.2%)	0.09
Loading dose clopidogrel	291/604 (48.2%)	242/444 (54.5%)	0.04	312/659 (47.3%)	222/391 (56.8%)	0.003
Statin	484/604 (80.1%)	349/444 (78.6%)	0.54	529/659 (80.3%)	305/391 (78.0%)	0.38
Beta-blocker	463/604 (76.7%)	336/444 (75.7%)	0.71	499/659 (75.7%)	301/391 (77.0%)	0.64
ACE-inhibitor	218/604 (36.1%)	174/444 (39.2%)	0.31	245/659 (37.2%)	147/391 (37.6%)	0.89
PPI	179/604 (29.6%)	117/444 (26.4%)	0.24	189/659 (28.7%)	107/391 (27.4%)	0.65
CCB	219/604 (36.3%)	175/444 (39.4%)	0.30	236/659 (35.8%)	158/391 (40.4%)	0.14
Oral antidiabetics	35/604 (5.8%)	35/444 (7.9%)	0.18	40/659 (6.1%)	31/391 (7.9%)	0.25
Coumadins	61/604 (10.1%)	47/444 (10.6%)	0.80	65/659 (9.9%)	43/391 (11.0%)	0.56
Laboratory Parameters						
Platelet count ($x10^9$)	267.8 ± 76.1	275.7 ± 83.4	0.13	271 ± 82.2	270.4 ± 74.4	0.85
WBC ($x10^9$)	8.1 ± 11.0	7.7 ± 2.3	0.48	8.0 ± 10.6	7.8 ± 2.4	0.57
Haemoglobin (mmol/L)	8.7 ± 2.6	8.3 ± 1.0	0.002	8.6 ± 2.6	8.4 ± 1.0	0.06

Procedural Parameters						
No.of stents implanted	938	703	0.93	1135	610	0.94
No.of lesions treated	809	639	0.08	896	556	0.17
Minimal Stent diameter (mm)	3.1 ± 0.9	3.1 ± 0.6	0.99	3.1 ± 0.9	3.1 ± 0.7	0.78
Total Stent length (mm)	28.3 ± 17.0	28.0 ± 16.7	0.70	28.0 ± 16.7	28.6 ± 17.3	0.56
Bifurcation lesion	17/604 (2.8%)	14/445 (3.1%)	0.75	16/659 (2.4%)	16/392 (4.1%)	0.13
Drug eluting stent	355/603 (58.9%)	239/444 (54.3%)	0.37	377/658 (57.3%)	219/387 (56.6%)	0.83
LAD	277/604 (45.9%)	229/445 (51.5%)	0.07	308/659 (46.7%)	197/392 (50.3%)	0.27
Graft	17/604 (2.8%)	16/445 (3.6%)	0.47	22/659 (3.3%)	11/392 (2.8%)	0.63

	VerifyNow P2Y12			Plateletworks		
Clinical parameters	NPR (n=646)	HPR (n=406)	p-value	NPR (n=344)	HPR (n=262)	p-value
Age (yrs)	63 ± 10.4	66 ± 10.6	<0.0001	63 ± 11.0	64 ± 10.1	0.35
BMI (kg/m2)	26.9 ± 3.7	27.7 ± 4.5	0.001	26.8 ± 3.7	27.6 ±4.0	0.03
Gender (m/f)	527/119	263/143	<0.0001	265/79	193/69	0.34
Hypertension	494/646 (76.5%)	318/406 (78.3%)	0.49	260/344 (75.6%)	201/262 (76.7%)	0.75
Hypercholesterolemia	521/646 (80.7%)	322/406 (79.3%)	0.60	273/344 (79.4%)	206/262 (78.6%)	0.83
Diabetes Mellitus	91/646 (14.1%)	103/406 (25.4%)	<0.0001	52/344 (15.1%)	57/262 (21.8%)	0.04
Family History	379/646 (58.5%)	256/406 (63.1%)	0.14	216/344 (62.8%)	149/262 (56.9%)	0.14
Current smoking	77/646 (11.9%)	39/406 (9.6%)	0.24	38/344 (11.0%)	22/262 (8.4%)	0.28
LVEF < 45%	88/646 (13.6%)	75/406 (18.5%)	0.03	55/344 (16.0%)	41/262 (15.6%)	0.91
Renal insufficiency	46/646 (7.1%)	36/406 (8.9%)	0.30	29/344 (8.4%)	17/262 (6.5%)	0.37
Prior MI	347/646 (53.7%)	226/406 (55.7%)	0.54	181/344 (52.6%)	139/262 (53.1%)	0.91
Prior PCI	212/646 (32.8%)	128/406 (31.5%)	0.66	120/344 (34.9%)	84/262 (32.1%)	0.47
Prior CABG	59/646 (9.1%)	48/406 (11.8%)	0.16	35/344 (10.2%)	32/262 (12.2%)	0.43
Medication						
Aspirin	589/646 (91.2%)	351/405 (86.7%)	0.02	317/344 (92.2%)	227/261 (87.0%)	0.04
Loading dose clopidogrel	320/646 (49.5%)	216/405 (53.3%)	0.23	155/344 (45.1%)	150/261 (57.5%)	0.002

Statin	518/646 (80.2%)	316/405 (78.0%)	0.40	266/344 (77.3%)	201/261 (77.0%)	0.93
Beta-blocker	493/646 (76.3%)	309/405 (76.3%)	0.99	261/344 (75.9%)	202/261 (77.4%)	0.66
ACE-inhibitor	238/646 (36.8%)	153/405 (37.8%)	0.76	121/344 (35.2%)	99/261 (37.9%)	0.49
PPI	177/646 (27.4%)	118/405 (29.1%)	0.54	84/344 (24.4%)	75/261 (28.7%)	0.23
CCB	242/646 (37.5%)	155/405 (39.3%)	0.79	146/344 (42.4%)	99/261 (37.9%)	0.26
Oral antidiabetics	35/646 (5.4%)	34/405 (8.4%)	0.06	19/344 (5.5%)	21/261 (8.0%)	0.22
Coumadins	62/646 (9.6%)	44/405 (10.9%)	0.51	23/344 (6.7%)	25/261 (9.6%)	0.19
Laboratory Parameters						
Platelet count ($x10^9$)	276.9 ± 85.6	263.7 ± 74.4	0.01	277.9 ± 87.2	270.1 ± 76.5	0.24
WBC ($x10^9$)	7.8 ± 2.6	8.1 ± 13.3	0.60	7.8 ± 2.8	8.6 ± 15.8	0.40
Haemoglobin (mmol/L)	8.7 ± 1.0	8.3 ± 3.2	0.02	8.7 ± 3.3	8.6 ± 0.9	0.35
Procedural Parameters						
No.of stents implanted	1018	628	0.14	533	403	0.61
No.of lesions treated	882	572	0.39	464	369	0.60
Minimal Stent diameter (mm)	3.1 ± 0.6	3.1 ± 1.1	0.58	3.0 ± 0.5	3.1 ± 0.5	0.10
Total Stent length (mm)	28.4 ±17.1	27.8 ±16.2	0.55	28.0 ± 16.0	28.4 ± 17.1	0.76
Bifurcation lesion	18/646 (2.8%)	15/406 (3.7%)	0.41	9/344 (2.6%)	6/262 (2.3%)	0.80
Drug eluting stent	371/642 (57.8%)	227/405 (56.0%)	0.57	191/344 (55.5%)	167/258 (64.7%)	0.10
LAD	311/646 (48.1%)	203/406 (50.0%)	0.56	169/344 (49.1%)	131/262 (50.0%)	0.83
Graft	16/646 (2.5%)	15/406 (3.7%)	0.26	8/344 (2.3%)	8/262 (3.1%)	0.58

	IMPACT-R			**IMPACT-R ADP**		
Clinical parameters	NPR (n=481)	HPR (n=429)	p-value	NPR (n=609)	HPR (n=296)	p-value
Age (yrs)	64 ± 11.1	65 ± 10.2	0.13	63 ± 10.6	66 ± 10.8	0.002
BMI (kg/m2)	27.4 ± 4.3	27.2 ± 3.9	0.45	27.2 ± 3.8	27.5 ± 4.6	0.28
Gender (m/f)	358/123	325/104	0.64	427/137	208/88	0.02
Hypertension	376/481 (78.2%)	332/429 (77.4%)	0.77	461/609 (75.7%)	243/296 (82.1%)	0.03

Hypercholesterolemia	387/481 (80.5%)	345/429 (80.4%)	0.99	482/609 (79.1%)	246/296 (83.1%)	0.16
Diabetes Mellitus	87/481 (18.1%)	79/429 (18.4%)	0.90	95/609 (15.6%)	70/296 (23.6%)	0.003
Family History	266/481 (55.3%)	278/429 (64.8%)	0.003	379/609 (62.2%)	160/296 (54.1%)	0.02
Current smoking	55/481 (11.4%)	43/429 (10.0%)	0.49	71/609 (11.7%)	24/296 (8.1%)	0.10
LVEF < 45%	64/481 (13.3%)	68/429 (15.9%)	0.27	93/609 (15.3%)	40/296 (13.5%)	0.48
Renal insufficiency	39/481 (8.1%)	33/429 (7.7%)	0.82	49/609 (8.0%)	23/296 (7.8%)	0.89
Prior MI	271/481 (56.3%)	229/429 (53.4%)	0.37	335/609 (67.4%)	162/296 (32.6%)	0.94
Prior PCI	144/481 (29.9%)	148/429 (34.5%)	0.14	198/609 (32.5%)	92/296 (31.1%)	0.67
Prior CABG	53/481 (11.0%)	47/429 (11.0%)	0.98	59/609 (9.7%)	41/296 (13.9%)	0.06
Medication						
Aspirin	426/481 (88.6%)	381/428 (89.0%)	0.83	543/609 (89.3%)	258/296 (87.2%)	0.34
Loading dose clopidogrel	246/481 (51.1%)	218/428 (50.9%)	0.95	299/609 (49.2%)	164/296 (55.4%)	0.08
Statin	381/481 (79.2%)	351/428 (82.0%)	0.29	495/609 (81.4%)	234/296 (79.1%)	0.40
Beta-blocker	360/481 (74.8%)	341/428 (79.7%)	0.08	473/609 (77.8%)	222/296 (75.0%)	0.35
ACE-inhibitor	170/481 (35.3%)	164/428 (38.3%)	0.35	230/609 (37.8%)	102/296 (34.5%)	0.32
PPI	136/481 (28.3%)	105/428 (24.5%)	0.20	158/609 (26.0%)	82/296 (27.7%)	0.58
CCB	178/481 (37.0%)	166/428 (38.8%)	0.58	246/609 (40.5%)	98/296 (33.1%)	0.03
Oral antidiabetics	32/481 (6.7%)	24/428 (5.6%)	0.51	34/609 (5.6%)	21/296 (7.1%)	0.38
Coumadins	51/481 (10.6%)	39/428 (9.1%)	0.45	56/609 (9.2%)	35/296 (11.8%)	0.22
Laboratory Parameters						
Platelet count ($x10^9$)	268.4 ± 83.6	275.8 ± 79.5	0.18	275.0 ± 82.9	265.7 ± 78.7	0.11
WBC ($x10^9$)	7.7 ± 2.2	7.7 ± 2.1	0.73	7.7 ± 2.2	7.7 ± 2.1	0.77
Haemoglobin (mmol/L)	8.4 ± 1.0	8.7 ± 3.1	0.09	13.8 ± 1.4	13.4 ± 5.9	0.41
Procedural Parameters						
No.of stents implanted	769	652		956	450	0.47
No.of lesions treated	658	595		836	505	0.73
Minimal Stent diameter (mm)	3.1 ± 1.1	3.1 ± 0.5	0.20	3.0 ± 0.5	3.2 ± 1.4	0.09
Total Stent length (mm)	28.7 ± 17.1	27.3 ± 15.9	0.19	28.3 ± 16.5	27.4 ± 16.2	0.44

Bifurcation lesion	8/481 (1.7%)	17/428 (4.0%)	0.03	17/609 (2.8%)	8/296 (2.7%)	0.94
Drug eluting stent	281/479 (58.7%)	234/426 (54.9%)	0.45	384/608 (3.2%)	188/292 (64.4%)	0.90
LAD	228/481 (47.4%)	209/428 (48.7%)	0.69	293/609 (48.1%)	142/296 (48.0%)	0.97
Graft	16/481 (3.3%)	11/428 (2.6%)	0.50	13/609 (2.1%)	14/296 (4.7%)	0.03

	PFA-100 COL/ADP			**INNOVANCE**		
Clinical parameters	NPR (n= 306)	HPR (n=506)	p-value	NPR (n=441)	HPR (n=147)	p-value
Age (yrs)	63 ± 10.2	64.5 ± 10.7	0.01	64 ± 10.5	66 ± 11.5	0.12
BMI (kg/m2)	27.0 ± 3.9	27.4 ± 4.2	0.24	27.0 ± 4.4	27.6 ± 4.3	0.18
Gender (m/f)	234/72	363/143	0.14	331/110	93/54	0.006
Hypertension	242/306 (79.1%)	378/506 (74.7%)	0.15	331/441 (75.1%)	93/147 (63.3%)	0.66
Hypercholesterolemia	253/306 (82.7%)	399/506 (78.9%)	0.18	325/441 (73.7%)	111/147 (75.5%)	0.82
Diabetes Mellitus	52/306 (17.0%)	98/506 (19.4%)	0.40	349/441 (79.1%)	115/147 (78.2%)	0.03
Family History	187/306 (61.1%)	301/506 (59.5%)	0.65	76/441 (17.2%)	37/147 (25.2%)	0.85
Current smoking	25/306 (8.2%)	59/506 (11.7%)	0.11	259/441 (58.7%)	85/147 (57.8%)	0.70
LVEF < 45%	36/306 (11.8%)	81/506 (16.0%)	0.10	47/441 (10.7%)	14/147 (9.5%)	0.03
Renal insufficiency	14/306 (4.6%)	44/506 (8.7%)	0.03	60/441 (13.6%)	31/147 (21.1%)	0.0005
Prior MI	174/306 (56.9%)	288/506 (46.9%)	0.99	26/441 (5.9%)	22/147 (15.0%)	0.89
Prior PCI	102/306 (33.3%)	144/506 (28.5%)	0.14	255/441 (57.8%)	84/147 (57.1%)	0.37
Prior CABG	41/306 (13.4%)	50/506 (9.9%)	0.12	124/441 (28.1%)	47/147 (32.0%)	0.50
				48/441 (10.9%)	19/147 (12.9%)	
Medication						
Aspirin	286/306 (93.5%)	430/506 (85.1%)	0.0004	399/441 (90.5%)	114/147 (77.6%)	<0.0001
Loading dose clopidogrel	141/306 (46.1%)	254/506 (50.3%)	0.24	206/441 (46.7%)	82/147 (55.8%)	0.06
Statin	249/306 (81.4%)	349/506 (78.0%)	0.25	350/441 (79.4%)	107/147 (72.8%)	0.10
Beta-blocker	223/306 (72.9%)	399/506 (79.0%)	0.05	332/441 (75.3%)	121/147 (82.3%)	0.08
ACE-inhibitor	118/306 (38.6%)	192/506 (38.0%)	0.88	165/441 (37.4%)	59/147 (40.1%)	0.56
PPI	88/306 (28.8%)	141/506 (27.9%)	0.80	137/441 (31.1%)	44/147 (29.9%)	0.80
CCB	114/306 (37.3%)	189/506 (37.4%)	0.96	164/441 (37.2%)	48/147 (32.7%)	0.32
Oral antidiabetics	22/306 (7.2%)	31/506 (6.1%)	0.56	26/441 (5.9%)	10/147 (6.8%)	0.69
Coumadins	19/306 (6.2%)	68/506 (13.5%)	0.001	43/441 (9.8%)	30/147 (20.4%)	0.0007

Laboratory Parameters						
Platelet count (x10^9)	276.0 ± 81.8	268.4 ± 82.5	0.21	264.5 ± 76.8	264.7 ± 75.8	0.98
WBC (x10^9)	7.3 ± 2.1	7.8 ± 2.3	0.009	7.5 ± 2.2	7.7 ± 2.8	0.47
Haemoglobin (mmol/L)	13.9 ± 5.8	13.8 ± 1.4	0.56	13.9 ± 4.8	13.1 ± 1.8	0.003
Procedural Parameters						
No.of stents implanted	456	799	0.11	668	225	0.54
No.of lesions treated	406	683	0.31	577	198	0.60
Minimal Stent diameter (mm)	3.0 ± 0.4	3.1 ± 1.0	0.19	3.1 ± 1.06	3.1 ± 0.5	0.77
Total Stent length (mm)	27.0 ± 15.2	27.9 ± 16.9	0.42	26.7 ± 15.8	26.8 ± 14.3	0.95
Bifurcation lesion	8/306 (2.6%)	12/506 (2.4%)	0..83	8/441 (1.8%)	4/147 (2.7%)	0.50
Drug eluting stent	196/306 (64. 0%)	304/506 (60.3%)	0.53	271/441 (61.5%)	94/147 (63.9%)	0.33
LAD	145/306 (47.0%)	254/506 (50.2%)	0.44	213/441 (48.3%)	78/147 (53.1%)	0.32
Graft	11/306 (3.6%)	13/506 (2.6%)	0.40	15/441 (3.4%)	5/147 (3.4%)	1.00

Baseline characteristics of the subpopulations according to the available platelet function measurements, divided in two group, according to HPR and NPR
HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity
Further abbreviations as in Appendix Table 1.

Definitions as in Table 1:

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.

Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.

Diabetes mellitus: According to the World Health Organization criteria

Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).

Renal insufficiency: Creatin > 120 µmol/L

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Chapter 3

Impact of platelet reactivity on clinical outcomes after percutaneous coronary intervention: collaborative meta-analysis of individual participant data

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ABSTRACT

Objective: To systematically evaluate the significance of platelet reactivity on clopidogrel treatment on adverse cardiovascular events using a collaborative meta-analysis using patient-level data for the VerifyNow P2Y12 assay.

Background: Clinical evidence has been controversial regarding the influence of clopidogrel on-treatment platelet reactivity and ischemic outcomes.

Methods: MEDLINE, Scopus, and the Cochrane library databases were searched through January 2010. A database containing individual patient-level time-to-event data was generated from identified studies. The primary outcome of interest was a composite of death, myocardial infarction, or stent thrombosis. Secondary outcomes included the incidence of: (1) death, (2) myocardial infarction, and (3) stent thrombosis.

Results: A total of six studies with 3,059 patients were included. In each study clopidogrel responsiveness was assessed using the same point of care assay after percutaneous coronary intervention (PCI). The primary endpoint occurred more frequently in higher-quartiles of P2Y12 reaction unit (PRU) values: quartile I, 5.8%; quartile II, 6.9%; quartile III, 10.9%; quartile IV, 15.8% (P<0.001). Taking quartile I as referent, the hazard ratio (95% CI; P-value) for the primary endpoint was: quartile II, HR 1.13 (0.72 - 1.78; P=0.60), quartile III, HR 1.82 (1.20 - 2.75; P=0.005), and quartile IV, HR 2.62 (1.78 - 3.87; P<0.001). On a continuous scale, every 10 unit increase in PRU was associated with a significantly higher rate of the primary endpoint (HR, 1.04; 95% CI, 1.03 – 1.06; P< .0001). According to receiver operating characteristic curve analysis, a PRU value of 230 appeared to best predict death, MI, or stent thrombosis (P<0.001). A PRU value ≥ 230 was associated with a higher rate of the composite primary endpoint (HR, 2.13; 95% CI, 1.64 - 2.77; P<0.0001), as well as the individual endpoints of death (HR, 1.68; 95% CI, 1.04 - 2.72; P=0.03), MI (HR, 2.07; 95% CI, 1.53 - 2.80; P<0.001), and stent thrombosis (HR, 2.65; 95% CI, 1.38 - 5.09; P=0.003).

Conclusions: In this collaborative meta-analysis, the level of on-treatment platelet reactivity according to the P2Y12 assay is associated with long-term cardiovascular events after PCI, including death, MI, and stent thrombosis.

INTRODUCTION

Dual anti-platelet therapy with aspirin and a thienopyridine is essential after percutaneous coronary intervention (PCI) with stent implantation.[1,2] However, significant inter-individual variability exists in clopidogrel-induced inhibition of platelet activation through the P2Y12 pathway. Several methods for assessment of on-clopidogrel treatment platelet reactivity have been developed.[3] While high on-treatment platelet reactivity has been associated with adverse cardiac events after PCI, the studies have had limited sample sizes, involved only single centers, and assessed only composite clinical endpoints. Therefore, we sought to investigate the relation of high on-treatment platelet reactivity with both composite and individual ischemic outcomes after PCI using a collaborative meta-analysis of patient-level data, and to derive a clinically meaningful cut-off platelet reactivity value to identify patients at risk of future ischemic events.

METHODS

Literature Search

We identified published studies assessing platelet reactivity using uniform methodology with a commercially available, point-of-care, cartridge-based assay (VerifyNow P2Y12 assay, Accumetrics, San Diego, CA). The following search criteria were used. Key words included the following in various combinations: platelet reactivity, clopidogrel, and VerifyNow. The databases searched included MEDLINE (1966 through January 2010), Scopus (1980 through January 2010), and the Cochrane Library (1993 through January 2010). We also searched conference proceedings of the American College of Cardiology, American Heart Association, Transcatheter Cardiovascular Therapeutics, European Society of Cardiology for "late breaking" presentations from 2005 - 2009. The references of review articles, meta-analysis, and evidence based-guidelines were reviewed by two authors (S.S.B., G.D.).[1,2,4] We did not use language restriction in the search.

Study Selection

To be included in this analyses studies needed to meet the following criteria: (1) use the VerifyNow P2Y12 test to assess platelet reactivity, (2) report the timing of assay performance in relation to PCI and clopidogrel loading, (3) report outcomes for death and myocardial infarction, and (4) report at least 30-day follow-up. Articles meeting the inclusion criteria were selected for further analysis. The investigators of the identified studies were contacted and each agreed to provide patient-level data.

Study Outcomes and Data Collection

The primary endpoint of this study was the composite of death, myocardial infarction, or stent thrombosis from the index percutaneous coronary intervention (PCI). The endpoints were defined according to the individual study protocols. Secondary endpoints included each one of the above components of the primary endpoint.

An electronic form containing the data elements to be completed for the patient level meta-

analysis was sent to all the principal investigators of the identified studies. Individual patient level data was provided for all six trials identified. The data requested for each enrolled patient included the date of the procedure, diabetes status, event status (including death, myocardial infarction, or stent thrombosis), age, gender, hypertension status, dyslipidemia, type of clinical presentation, stent type, and date of last follow-up. Any queries were resolved and the respective study investigators verified the final database entries.

PCI and Antiplatelet Management

All interventions were performed according to local standards. The type of stent implanted was left to the discretion of the operator in all studies. All patients received one clopidogrel loading dose of 300-600 mg followed by a daily dose of 75 mg. Aspirin 100 to 325 mg orally was administered post-procedure. Anticoagulation with either heparin or bivalirudin was at the discretion of the operator.

Platelet Reactivity Assessment

The time of blood withdrawal for P2Y12 testing was at the time of PCI in patients pre-treated with clopidogrel or at least 1 day after the clopidogrel loading dose. A uniform testing method for clopidogrel responsiveness was selected in order to eliminate the variability in assessment of platelet reactivity by different hematologic assays. Platelet reactivity testing to clopidogrel therapy was performed using the VerifyNow P2Y12 assay (Accumetrics, San Diego, CA.). This method has been approved for human use by the U.S. Food and Drug Administration. This assay is a turbidimetry-based optical detection device that measures platelet-induced aggregation in a system containing fibrinogen-coated beads. The P2Y12 assay contains 20 μM of ADP as the platelet agonist and 22 nmol of prostaglandin E1, to reduce the contribution of ADP binding to P2Y1 receptors. The instrument measures platelet-induced aggregation of the beads as an increase in light and expresses the results as P2Y12 reaction units (PRU).

Statistical Methods

Categorical variables were compared by the Chi-square or Fisher's exact test. Continuous variables are reported as mean ± standard deviation and were compared by unpaired *t*-tests. For variables that were not normally distributed (e.g. PRU quartiles), the Wilcoxon test was used for comparing two groups, and the Kruskal-Wallis for > 2 groups.

Time-to-event data are reported and displayed using the Kaplan-Meier method with comparisons between groups performed using the log-rank test. Cumulative survival curves by PRU quartiles were constructed by the Kaplan-Meier method. Quartiles II, III, and IV were compared to quartile 1 with the log-rank test. In the survival analyses, adjustments for multiple comparisons were performed by applying the Sidák correction to the raw p-values.[5]. Analyses were truncated at 2-years of follow-up due to the small number of patients with available data thereafter. Cox proportional hazards models were also generated for the primary efficacy and safety outcomes.

The proportionality assumption was tested using log(-log) plots and Schöenfeld residuals; the assumption was satisfied by both tests.

A landmark analysis was used to determine if there were long-term differences in the primary endpoint between groups with normal- vs. high-on treatment platelet reactivity after excluding peri-procedural events. In this analysis, all patients with events within the first three days post-PCI were excluded.

Logistic regression was used to generate a receiver-operating curve for the PRU values and the primary endpoint. The area under the curve (AUC) or c-statistic was determined from this model as was the optimal cut point; the latter was determined by the PRU value that maximized the following relationship: sensitivity – (1-specificity). Model goodness of fit was tested and satisfied by the Hosmer-Lemeshow goodness of fit test. The robustness of the PRU threshold value was also assessed in sensitivity analyses. The cohort was randomly divided into a derivation and validation dataset, with 50% of the sample distributed to each dataset. In the derivation dataset, bootstrap estimates (sampling with replacement) of the PRU threshold were calculated for 100 iterations, yielding the best average cutoff and 95% confidence interval. For estimates of standard errors and normal approximation confidence intervals, 100 bootstrap replications are generally adequate. Next, Kaplan-Meier failure estimates and hazard ratios were calculated using the PRU threshold in the derivation and validation cohorts.

Subgroups for further analyses were specified a priori and included: age, gender, diabetes status, stent type, and acute coronary syndrome presentation. The Cochrane Q statistic and the I^2 statistic were used to assess the heterogeneity across trials. A Cochrane Q statistic with a p-value ≤ 0.1 was considered significant. The I^2 statistic was used to measure the consistency among trials with values of 25, 50, and 75% showing, respectively, low, moderate, and high heterogeneity. A funnel plot was used to assess for the presence of publication and other reporting biases by plotting the standard error against the log risk ratio. Using Egger's regression method, we examined the association between the study size and estimated treatment.[6]

The P-value threshold for statistical significance was set at 0.05. Analyses were conducted by S.S.B in Stata 10.1 (Stat Corp., College Station, TX) and SAS 9.2 (SAS Institute., Cary, NC). The study was performed in accordance to the recommendations set forth by the Quality of Reporting of Meta-Analysis (QUOROM) and the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) work groups.[7,8] All authors have read and agree to the manuscript as written.

RESULTS

Eight studies were identified and six were included in the pooled analysis. Follow-up in one study was limited to in-hospital events and therefore it did not meet inclusion criteria.[9] We also excluded one study where testing was performed in an unspecified time frame shortly after oral clopidogrel loading dose was administered.[10] The authors of all six studies that met inclusion criteria provided patient level data for analysis[11-16] Data on death and myocardial infarction were available from all

six studies; stent thrombosis data were available from five of the studies. Data were collected prospectively in each of the studies included. Endpoints were adjudicated by an independent endpoints committee in two of the studies.[11,16] When studies included treatment arms or groups treated with antiplatelet therapies other than clopidogrel and aspirin, we only included patients receiving the combination of clopidogrel and aspirin.[12,13]

Study characteristics are shown in **Table 1**. The mean age (SD) of the cohort was 66 (10) years, 68% were male, 24% were diabetic, 74% had hypertension, 64% had dyslipidemia, and 20% were smokers. The time of blood withdrawal for P2Y12 testing was at the time of PCI in patients pre-treated with clopidogrel or at least 1 day after the clopidogrel loading dose. The distribution of PRU values by study and quartile is shown in **Figure 1**. The mean (SD) platelet reactivity of the full cohort was 196.5 (84.5) PRU and the median (IQR) was 200 (121) PRU. The median values were comparable between studies, except for the study by Valgimigli et al., which had the lowest median PRU value

Table 1. Study Characteristics

	Breet et al	Marcucci et al.	Kim et al.	Patti et al.	Price et al.	Valgimigli et al
Study year	2010	2009	2010	2008	2008	2010
Sample size	1069	683	361	160	317	468
Age, mean (SD), y	64 ± 11	68 ± 9	63 ± 9	66 ± 9	67 ± 11	67 ± 9
Women, No.(%)	267 (25.0%)	172 (25.2%)	96 (26.9%)	31 (19.4 %)	70 (22.1%)	119 (25.4%)
Acute coronary syndrome, No.(%)	199 (18.7%)	178 (26.1%)	111 (30.8%)	55 (33.4%)	93 (29.3%)	111 (23.7%)
Clopidogrel loading dose	0 (0%)	683 (100%)	137 (38.0%)	87 (54.4%)	20 (6.3%)	152 (32.5%)
Drug-eluting stent use, No. (%)	675 (63.1%)	121 (17.7%)	361 (100%)	41 (26%)	317 (100%)	314 (67%)
Clopidogrel loading dose	300 mg if≥24h before PCI, else 600 ≥4h before PCI	600 mg	300 mg if≥24h before PCI, else 600 ≥4h before PCI	600 mg	600 mg	600 mg
Minimum duration of clopidogrel therapy, months	12	12	6	12	6	6
Timing of platelet reseponsivesnness	At time of PCI	24 hrs post PCI	24-48 hrs post PCI			
Primary endpoint	All cause death, MI, stent thrombosis, ischemic stroke	Cardio vascular death, MI	Cardio vascular death, MI, ischemic stroke, TLR	Cardio vascular death, MI, TVR	Cardio vascular death, MI, stent thrombosis	All-cause death, MI, ischemic stroke
Duration of follow-up, months	12	12	6	1	6	12

* Duration of clopidogrel was at least one month and 12-months in patients presenting with ACS or treated with DES. ** 1 month minimum in patients treated with BMS.

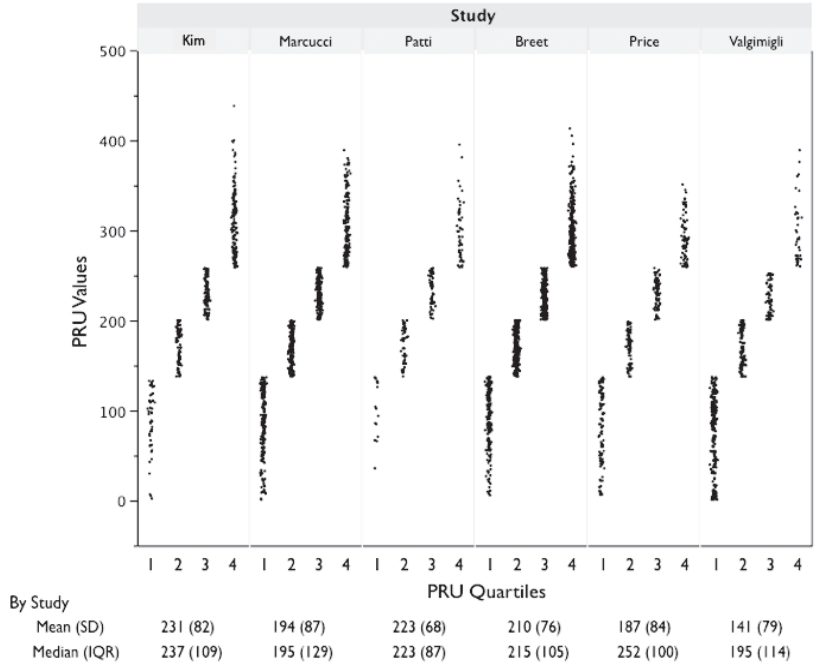


Figure 1. Distribution of P2Y12 reaction units (PRU) by study and quartile.

(P< .001).(12) Quartile I represents patients with the lowest on-treatment platelet reactivity whereas quartile IV represents those with the highest on-treatment platelet reactivity. In the full study cohort, the mean PRU values (SD) for quartiles I to IV were 84.5 (37.3), 171 (18.7), 229.7 (16.7), 301 (32.9), respectively (P< .001). The respective median PRU values (IQR) were 92 (57), 172 (33), 229 (28), and 294 (49), P< .001.

Heterogeneity & Small Study Effects

Prior to performing the pooled analysis, we assessed heterogeneity across studies. There was no evidence for heterogeneity between studies by either the Cochrane Q statistic (P= .56) or the I^2 statistic (I^2 = 0%). Also, visual inspection of the funnel plot did not reveal asymmetry in the studies (**see appendix-figure 1**). In support, Egger's regression test was not statistically significant for a small study effect or publication bias (P= .62).

Main Outcomes

The long-term clinical outcomes for the primary composite endpoint of death, MI, or stent thrombosis are shown in **Figure 2**. Multiple pair-wise comparisons were performed, taking quartile I as referent. All pair-wise comparisons were adjusted for multiple testing as previously described. The event rates were similar between quartiles I and II (P= .97). However, the event rates in quartiles III and IV were significantly greater compared to quartile I(P=0.02 and P<0.001, respectively). The

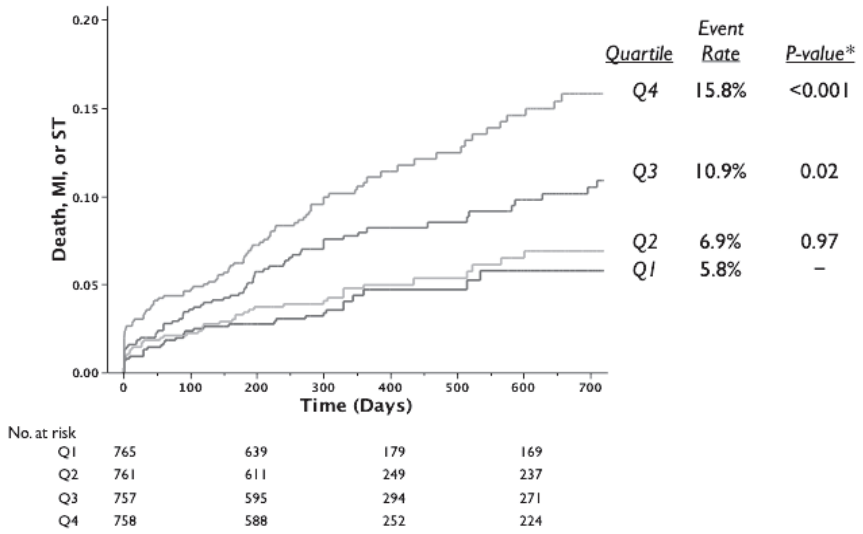


Figure 2. Kaplan-Meier curve for probability of death, myocardial infarction, or stent thrombosis by PRU quartiles at 2-years. Quartile 1, the group with the lowest PRU values, was taken as referent. Pair-wise comparisons were then made with the referent group and adjusted for multiple comparisons.
* Log-rank p-values were adjusted for multiple comparisons.

hazard ratios (95% CI) for the primary endpoint for quartiles II, III, and IV compared to quartile I were 1.13 (0.72 - 1.78), 1.82 (1.20 - 2.75), and 2.62 (1.77 - 3.87), respectively. When PRU values were analyzed on the continuous scale, there remained a statistically significant association. There was a 4% increase in the primary endpoint for every 10-unit increase in PRU (HR, 1.04; 95% CI, 1.03 – 1.06; P< .0001).

The rate of death was not significantly different across PRU quartiles, although the highest rate of death occurred in quartile IV (**Figure 3a**). The pair-wise comparisons, taking quartile I as referent, were not significantly different for quartile II (P= .97), quartile III (P= .92), and quartile IV (P= .30). The hazard ratios (95% CI) for mortality for quartiles II, III, and IV compared to quartile I were 0.84 (0.39 - 1.81), 1.24 (0.62 - 2.50), and 1.67 (0.85 - 3.23), respectively.

The rate of myocardial infarction differed significantly between quartiles (**Figure 3b**). The pair-wise comparison, taking quartile I as referent, was similar for quartile II (P=0.78) but the event rate was significantly greater in quartile III (P= .007) and quartile IV (P< .001). The hazard ratios (95% CI) for MI for quartiles II, III, and IV compared to quartile I were 1.34 (0.78 - 2.30), 2.23 (1.36 - 3.64), and 2.93 (1.82 - 4.71), respectively.

The rate of stent thrombosis by PRU quartile is shown in **Figure 3c**. The event rate was significantly greater in quartile IV compared to quartile I, 3.4% vs. 0.4%, respectively (P= .002). However, there was no significant difference between quartile II (P= .67) and quartile III (P= .59) compared to quartile I. The corresponding hazard ratios (95% CI) for quartiles II, III, and IV, taking quartile I as referent, were 3.26 (0.68 – 15.69), 3.11 (0.65 – 14.96), and 7.48 (1.72 – 32.52), respectively.

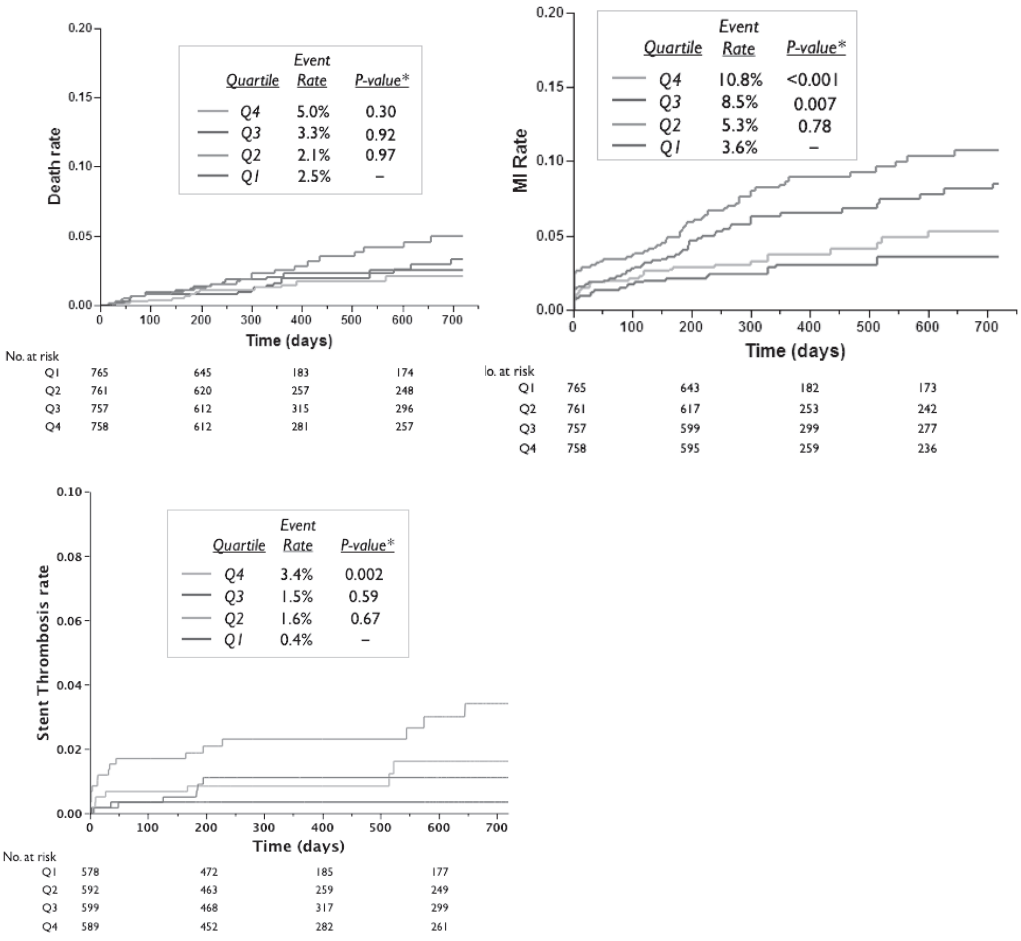


Figure 3. Kaplan-Meier curve for probability of death (**a**), myocardial infarction (**b**), and stent thrombosis (**c**) by PRU quartiles. Quartile 1 (Q1) was taken as referent.

* Log-rank p-values were adjusted for multiple comparisons.

Threshold Analysis

Using logistic regression, a receiver operating characteristic curve was able to distinguish between patients with and without subsequent ischemic events (area under the curve 0.61; 95% CI, 0.57 to 0.65; P< .001). The optimal cut-off value to predict death, myocardial infarction, or stent thrombosis was a PRU value of 230 with corresponding sensitivity, specificity, positive predictive value, and negative predictive values of 55%, 65%, 11%, and 95%, respectively. Patients with PRU values ≥ 230 were categorized as having high on-treatment platelet reactivity and for values < 230 as having normal on-treatment platelet reactivity. There were no differences in patients with or with high on-treatment platelet reactivity for female gender (36% vs. 39%; P=0.11), hypertension (32% vs, 38%; P=0.22), dyslipidemia (37% vs. 37%; P=0.92), or an acute coronary syndrome (36% vs. 38%; P=0.54).

However, diabetes was significantly more frequent in subjects with high on-treatment platelet reactivity, 30% vs. 21% (P< .001).

The Kaplan-Meier curve for the composite primary endpoint of death, myocardial infarction, or stent thrombosis is shown in **Figure 4a**. Patients with high on-treatment platelet reactivity had a significantly higher event rate for the primary endpoint, 14.7% vs. 7.0% (P< 0.001); the corresponding hazard ratio for the high vs. normal on-treatment platelet reactivity was 2.13 (95% CI, 1.64 - 2.77; P< .0001) (**Table 2**). When effects on individual endpoints were examined, a PRU value ≥ 230 was associated with a significantly higher rate of mortality (HR, 1.68; 95% CI, 1.04 - 2.72; P= .03), myocardial infarction (HR, 2.07; 95% CI, 1.53 - 2.80; P< .001), and stent thrombosis (HR, 2.50; 95% CI, 1.31 - 4.79; P= .005).

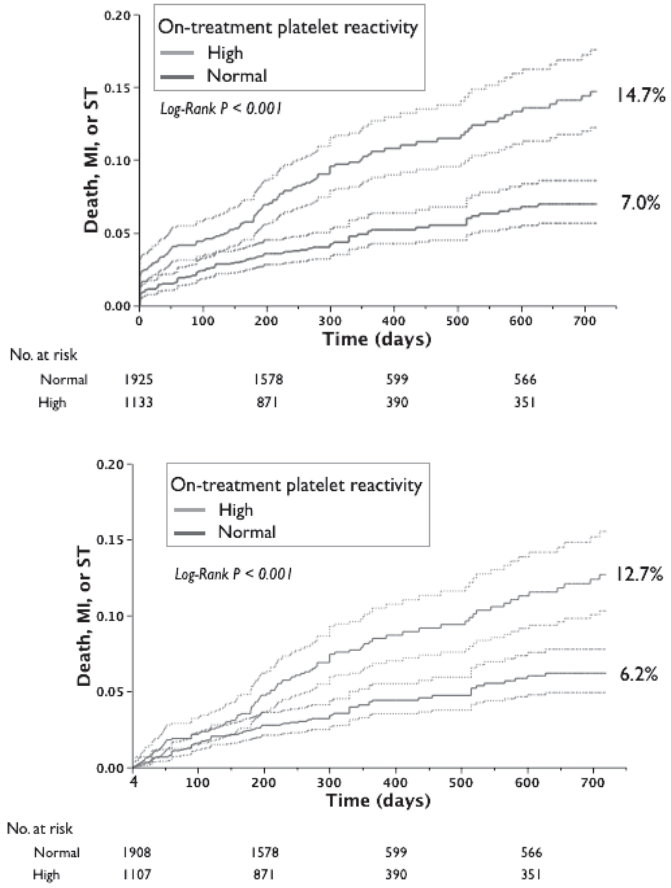


Figure 4. Kaplan-Meier curve for probability of death, myocardial infarction by platelet reactivity. High on-treatment platelet reactivity was defined as PRU values ≥ 230 and normal as PRU values < 230. Dashed lines represent the 95% confidence intervals for each group. (**a**) Full cohort. (**b**) Landmark analysis starting day 4 post PCI.

Table 2. Event rates according to on-treatment platelet reactivity status.

	On-treatment Platelet reactivity, n/N (%)			
	PRU ≥ 230	PRU<230	Hazard ratio (95%-Confidence Interval)	p
Death/MI/Stent thrombosis	124/1133 (14.7%)	103/1925 (7.0%)	2.13 (1.64-2.77)	<0.0001
Death	33/1133 (4.5%)	34/1924 (2.5%)	1.68 (1.04-2.72)	0.03
MI	92/1133 (10.3%)	78/1925 (5.2%)	2.07 (1.53-2.80)	<0.0001
Stent thrombosis	26/825 (3.8%)	14/1087 (1.5%)	2.50 (1.31-4.79)	0.005

n, number of events; N, number of subjects per group; %, rates are Kaplan-Meier estimates

In sensitivity analyses, we divided the cohort into derivation and validation datasets. Using the derivation dataset, the bootstrap (sampling with replacement) analysis comprising 100 iterations yielded a PRU threshold value similar to the main analysis; the average best PRU cutoff was 231 (95% CI: 190-272) (**see appendix-table 1**). In the derivation dataset, the Kaplan-Meier failure rate in subjects above the 231 threshold was 14.1% compared to 7.1% in those below the cutoff (P=0.0001); the corresponding hazard ratio (95% CI) was 2.07 (1.50-2.86; P<0.001) (**see appendix-figure 2**). The performance of the PRU threshold was then evaluated in the validation dataset. The Kaplan-Meier failure estimate for the event rate remained qualitatively unchanged; it was 14.8% in patients above the threshold and 7.3% in those below the threshold (P=0.0002); the hazard ratio (95% CI) was 2.00 (1.38-2.91; p<0.001) (**see appendix-figure 3**).

Subgroup Analysis

The event rates for pre-specified subgroups of gender, age, diabetes, and clinical presentation were also determined (**Table 3**). High on-treatment platelet reactivity was associated with higher rates of death, MI, or stent thrombosis for men and women, for those > 65 yrs or ≤ 65 yrs, and for persons with or without an acute coronary syndrome presentation. However, for diabetes, assessment of platelet reactivity was associated with a significantly higher event rate in the cohort without diabetes only. The hazard ratio for subjects with high vs. normal on-treatment platelet reactivity was 2.49 (95% CI, 1.84 - 3.39; P< .0001) for non-diabetics and 1.30 (95% CI, 0.79 - 2.15; P= .32) for diabetics ($P_{interaction}$ = .03).

In post-hoc analyses, we investigated the relationship between type of stent and adverse cardiovascular outcomes. For the composite primary endpoint of death, MI, or stent thrombosis, the hazard ratio for high vs. normal on-treatment reactivity for patients treated with bare metal or drug-eluting stents was 2.49 (95% CI, 1.44-4.32; P= .001) and 2.27 (95% CI, 1.57-3.03; P< .001), respectively.

Table 3. Selected Subgroup Analysis by On-Treatment Platelet Reactivity Status.

	On-treatment Platelet reactivity, n/N (%)[a]				
Death/MI/Stent thrombosis	PRU ≥ 230	PRU<230	Hazard ratio (95%-Confidence Interval)	P[b]	P[c]
Male	87/748 (16.2%)	70/1324 (7.1%)	2.37 (1.73-3.24)	<0.0001	0.27
Female	37/385 (12.1%)	33/601 (6.4%)	1.73 (1.08-2.78)	0.02	
Age > 65 years	77/697 (14.6%)	64/1022 (8.4%)	1.84 (1.32-2.56)	0.0003	0.20
Age ≤ 65 years	47/436 (14.7%)	39/904 (5.5%)	2.56 (1.69-4.00)	<0.0001	
Diabetes, yes	32/346 (13.3%)	29/401 (10.9%)	1.30 (0.79-2.15)	0.32	0.03
Diabetes, no	92/787 (15.3%)	74/1522 (6.2%)	2.49 (1.84-3.39)	<0.0001	
Acute coronary syndrome, yes	14/144 (11.1%)	11/256 (4.3%)	2.97 (1.37-6.45)	0.006	0.64
Acute coronary syndrome, no	92/743 (12.4%)	64/1232 (5.2%)	2.47 (1.79-3.40)	<0.0001	

[a] n, number of events; N, number of subjects per group; %, rates are Kaplan-Meier estimates
[b] P value for the treatment comparison within the subgroup
[c] P value is testing the treatment x subgroup interaction

Sensitivity & Influence Analysis

Since peri-procedural myocardial infarction was included in certain studies, we performed a sensitivity analysis where all events in the first three days post PCI were censored. In this 3-day landmark analysis, the results were qualitatively similar to those in the main analysis. The rate of the composite primary endpoint was significantly greater in the high on-treatment platelet reactivity group, 12.7% vs. 6.2%, respectively (P< .001) (**Figure 4b**). The corresponding hazard ratio (95% CI) was 2.01 (1.50 - 2.68), for the composite primary endpoint (P< .0001). For the components of the primary endpoint, the hazard ratio in the landmark analysis was 1.70 (95% CI, 1.04 - 2.75; P= .03) for death, 1.88 (95% CI, 1.33 - 2.66; P= .0004) for MI, and 2.31 (95% CI, 1.16 - 4.59; P= .02) for stent thrombosis.

In influence analysis, we investigated the impact of the largest study in the cohort. When the study by Breet et al., which enrolled 1,069 patients, was removed from the analysis, the primary results were unchanged. The hazard ratio for the primary endpoint, taking quartile I as referent, was 1.03 (95% CI, 0.59-1.82; P= .92) for quartile II, 1.75 (95% CI, 1.04-2.95; P= .035) for quartile III, and 2.15 (95% CI, 1.31-3.52; P= .003) for quartile IV. For patients with a PRU value ≥ 230 vs. < 230, the results were also similar to the main analysis; the hazard ratio for death, MI or stent thrombosis for PRU ≥ 230 vs. < 230 was 1.83 (95% CI, 1.27-2.62; P= .001).

DISCUSSION

We performed a patient-level pooled meta-analysis of six prospective studies that quantified on-clopidogrel platelet reactivity with a uniform methodology in patients undergoing PCI. The

principal finding of our study is that higher on-treatment platelet reactivity measured using the VerifyNow P2Y12 assay was predictive of long-term ischemic events.

Main outcomes. We observed a higher event rate of the composite primary endpoint of death, MI, or stent thrombosis for increasing levels of on-treatment platelet reactivity through 2-years of follow-up. Importantly, the highest quartile of PRU values (i.e. highest level of on-treatment platelet reactivity), was also associated with a significant increase in the individual rates of non-fatal MI, and stent thrombosis. The event rate for the primary endpoint in the highest quartile of PRU values was significantly greater compared to the lowest quartile, 15.8% vs. 5.8% (hazard ratio, 2.62; 95% CI, 1.77 - 3.87; P< .001). Quartile III was also associated with a higher rate of death, MI, and stent thrombosis when compared to quartile I (P= .005). For the primary or secondary endpoints there were no significant differences between quartiles I and II. Therefore, our observations support a threshold effect for the relationship between on-treatment reactivity and ischemic events post-PCI. We identified a potential cut-off value of a PRU > 230 for high on-treatment platelet reactivity and the composite endpoint of death, MI, or stent thrombosis post-PCI using receiver-operator characteristics curve analysis.

Stent thrombosis. Stent thrombosis remains a vexing problem associated with a high rate of morbidity and mortality post-PCI. In both the quartile and threshold analysis using the 230 PRU cut-off, we observed a significantly higher rate of stent thrombosis in persons with higher on-treatment platelet reactivity. The stent thrombosis rate using the threshold value of 230 PRU was 3.8% vs. 1.5%; the corresponding hazard ratio was 2.50 (95% CI, 1.31 – 4.79; P= .005). A similar observation was made in a smaller cohort study using ADP mediated platelet aggregation. In that study, non-responsiveness to clopidogrel was associated with a hazard ratio of 3.08 (95% CI, 1.32 – 7.16; P= .009) for stent thrombosis.[17] The ability of a single antiplatelet aggregation assessment post-PCI to predict stent thrombosis may have important clinical implications.

Sensitivity analyses & subgroups. We performed a landmark analysis in an attempt to better understand the importance of platelet P2Y12 reactivity testing post-PCI with respect to longer-term outcomes. There remained a significant association between on-treatment platelet reactivity and long-term out-of-hospital ischemic events when events in the first 3-days post-PCI were excluded. This observation further supports the relationship between high on-treatment platelet reactivity identified around the time of PCI and the risk of long-term adverse cardiovascular events.

We performed several pre-specified sub-group analyses to determine whether the effect of high on-treatment reactivity was consistent across the population studies. We observed similar rates of the composite of death, MI, or stent thrombosis in the those > 65 or ≤ 65 years of age; in women and men; in the presence of or absence of an acute coronary syndrome presentation; and in patients treated with drug eluting or bare-metal stents. Interestingly, we observed a potential interaction in the diabetic subgroup. Quantifying platelet reactivity post-PCI appeared significantly predictive in patients without diabetes but did not reach statistical significance in those with diabetes. This

raises the questions of whether risk stratification with platelet function testing may be especially important in patients without diabetes; this potential interaction warrants further investigation in future studies.

Threshold analyses. The threshold PRU value of 230, obtained using the full cohort, is associated with an increase in death, myocardial infarction, and stent thrombosis after PCI. To assess the robustness of this value, the full cohort was divided into derivation and validation datasets. The PRU threshold from this analysis, 231, was qualitatively similar to the 230 cutoff. The composite primary outcome was validated internally using the derivation dataset by sampling with replacement for 100 iterations. External validation was performed using the validation cohort. Kaplan-Meier failure rates and corresponding hazard ratios were similar in the derivation and validation cohorts supporting the PRU threshold identified.

Despite the statistical significance and consistency of the above findings, the AUC or c-statistic of the assay used was modest. The AUC in the component studies ranged from 0.61 to 0.80 and in the pooled analysis was 0.61. Receiver operating characteristic curve analysis is frequently used to describe diagnostic test performance, with the AUC being indicative of the discriminatory ability of the test in question compared to a gold standard.[18] While diagnostic tests used to identify patients with a specific disease often have high AUCs, tests used to identify patients at risk of developing a clinical endpoint (like that examined in the present study) often have modest AUCs. Prognostication frequently involves estimating risk or the probability of a future event, adding a stochastic element, distinguishing this task from diagnosis.[19] Methods are not well developed for time to event data; therefore, AUC values from predictive models should be interpreted cautiously.[20] For comparison, in a comprehensive assessment by the Agency for Health Care Quality and Research the AUC for B-type natriuretic peptide (BNP) and NT-proBNP were 0.57 - 0.88 across several studies, not notably different from the AUC range observed using the VerifyNow P2Y12 assay in the present study.[21] Forgoing an attempt at a dichotomous separation, there remains a strong relationship between PRU values on the continuous scale and the primary endpoint. The rate of death, MI, or stent thrombosis increased by 4% for every 10 unit increase in PRU ($P < .0001$); and there was a strong association observed using the 230 PRU cut-off with the "hard" clinical endpoints of death, non-fatal MI, and stent thrombosis with an absolute risk difference of 7.7%.

Future directions. Several antiplatelet strategies may potentially be used in patients with high on-treatment platelet reactivity, including increasing the clopidogrel dose or switching to alternative P2Y12 inhibitors.[22,23,24,25] The clinical benefit of such "personalized anti-platelet therapy" has been examined in small single center trials.[22,26] Several multicenter randomized trials are planned or are currently enrolling patients to test different antiplatelet strategies using platelet function testing, including GRAVITAS (NCT00645918), TRIGGER-PCI (NCT00910299), ARTIC (NCT00827411), and DANTE (NCT00774475).

Limitations

In the present study, data regarding CYP2C19 genotype were not available, and therefore the impact of genotype on platelet function and clinical outcomes could not be assessed. Also, we were not able to assess bleeding complications because this outcome was not consistently included in the trials we included in our analysis. Bleeding complications are very important with respect to mortality risk; however, their clinical importance in relation to oral antiplatelet therapy has been recognized after completion of the studies we analyzed.[27]

CONCLUSION

The results of this study show that high on-treatment platelet reactivity around the time of PCI is associated with long-term cardiovascular events including death, MI, and stent thrombosis. These findings were consistently observed in landmark, sensitivity, and influence analyses. Also, using the P2Y12 point-of-care assay, a PRU value of ≥ 230 was associated with higher rates of death, MI, or stent thrombosis. Future randomized controlled trials investigating the role of oral antiplatelet therapy guided by P2Y12 reactivity testing will provide insight into effective therapeutic interventions for patients with high on-treatment platelet reactivity.

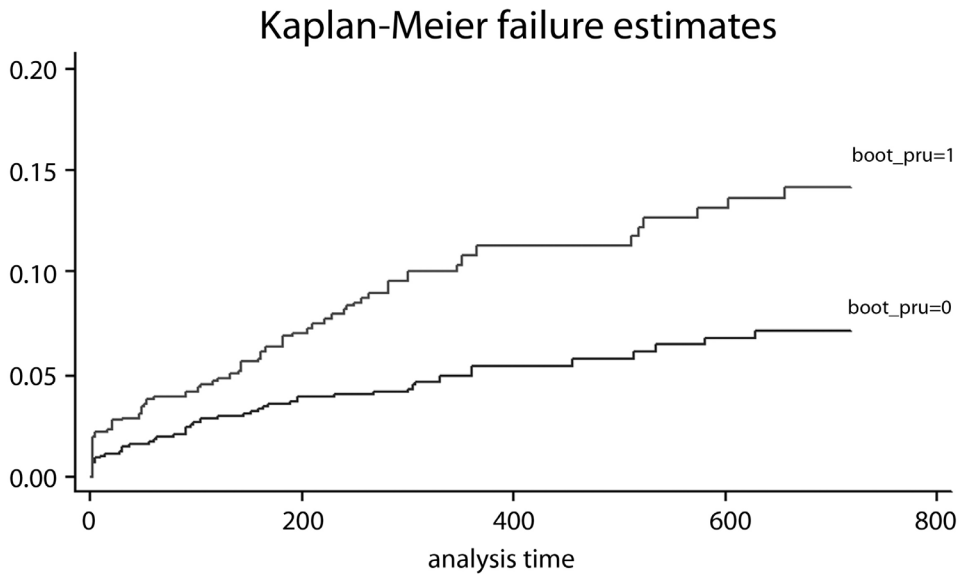
APPENDIX

Analysis outline*:*

The cohort was randomly divided into a derivation and validation dataset, with 50% of the sample distributed to each dataset. The derivation dataset was used to calculate a PRU threshold for the outcome of death, myocardial infarction, and stent thrombosis. The threshold value for the derivation cohort was determined by finding the PRU value that maximized the relationship: sensitivity - (1 - specificity). Bootstrap estimates (sampling with replacement) of the corresponding PRU threshold were calculated for a total of 100 iterations, yielding the average best cutoff and 95% confidence interval.

Derivation:

The bootstrap estimate of the cut off value in the derivation cohort was 231 (95% CI: 190-272).

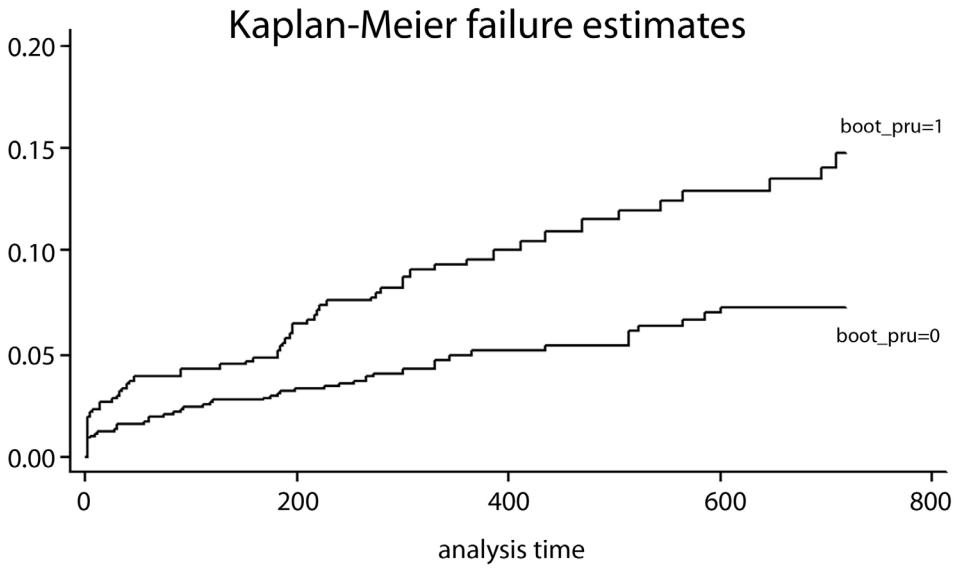


The Kaplan-Meier event rates were calculated in the derivation cohort based upon the cutoff value obtained from the bootstrap analysis. The event rate in subjects above the 231 cutoff was 14.1% compared to 7.1% in those below the cut off. The log rank P-value was 0.0001.

The hazard ratio and 95% confidence interval were also calculated in the derivation dataset using bootstrap sampling and estimation from a Cox proportional hazards model. The observed hazard ratio for the primary endpoint for patients above verses below the threshold value of 231 was 2.07 (95% CI: 1.50-2.86; P <0.001).

Validation:

The performance of the cutoff value of 231 was evaluated in the validation data set. The Kaplan-Meier estimate for the event rate was 14.8% in patients above the threshold and 7.3% in patients below the threshold. The log rank P value was 0.0002.



The bootstrapped hazard ratio with 100 replications was 2.00 (1.36-2.94; P <0.001) in the validation cohort.

For comparison, the hazard ratio in the validation cohort *without* bootstrapping was 2.00 (1.38-2.91; p<0.001).

Summary table:

	Derivation cohort	Validation cohort	Full Cohort
PRU Threshold	231*	231**	230
Kaplan-Meier estimates	14.1% vs. 7.1%	14.8% vs. 7.3%	14.7% vs. 7.0%
Log rank test	0.0001	0.0002	<0.001
HR (95% CI)	2.07 (1.50-2.86)	2.00 (1.36-2.94)	2.13 (1.64 – 2.77)
P value	<0.001	<0.001	<0.001

* bootstrapping (with replacement), 100 replications
** value determined from derivation cohort bootstrap analysis.

Notes:

- All analyses were performed with 100 bootstrap replications, which is generally adequate for estimates of standard error and thus are adequate for normal-approximation confidence intervals.
- Analyses were performed in STATA 10.1.

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Chapter 4

High on-aspirin platelet reactivity as measured with aggregation based, COX-1 inhibition sensitive platelet function tests is associated with the occurrence of atherothrombotic events

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ABSTRACT

Background: High on-aspirin platelet reactivity (HAPR) is associated with atherothrombotic events following percutaneous coronary intervention (PCI). The aim of the present study is to identify the platelet function test sensitive for platelet COX-1-inhibition that best predicts atherothrombotic events.

Methods and results: 951 consecutive patients on dual antiplatelet therapy undergoing elective PCI were enrolled. On-aspirin platelet reactivity was measured in parallel by arachidonic acid (AA)-induced light transmittance aggregometry (LTA), the VerifyNow® Aspirin-assay, the IMPACT-R and the PFA-100® Collagen/Epinephrine-cartridge. Cut-offs for HAPR were established by receiver-operator characteristic (ROC) curve analysis.

At one-year follow-up, the composite of all-cause death, non-fatal acute myocardial infarction, stent thrombosis and ischemic stroke occurred more frequently in patients with HAPR when assessed by LTA (10.1% vs 6.0%, p=0.020 [n=925]) and VerifyNow® (13.3% vs 5.9%, p=0.015 [n=422]). The VerifyNow® ASA assay (AUC=0.78) and to a lesser extent AA-induced LTA (AUC=0.73) added significantly to a model consisting of clinical and procedural risk factors in predicting atherothrombotic events. In contrast, the IMPACT-*R* (n=791) and the PFA Collagen/Epinephrine (n=719) were unable to discriminate between patients with and without primary endpoint at one-year follow-up. None of the platelet function tests were able to identify patients at risk for bleeding.

Conclusions: AA-induced LTA and the VerifyNow® ASA test were able to identify aspirin-treated patients undergoing PCI with stenting at risk for atherothrombotic events. The VerifyNow® Aspirin-assay had the highest predictive accuracy. None of the tests were able to identify patients at higher risk of bleeding.

INTRODUCTION

Aspirin is the most widely used drug and exerts its effects by the irreversible inhibition of platelet cyclooxygenase (COX)-1, a key-enzyme in the conversion of arachidonic acid (AA) to the potent platelet agonist thromboxane (TX)A_2.[1] However, throughout the last two decades it has become apparent that the individual biological response to low dose aspirin is heterogeneous.[2,3] Patients with a low response to aspirin as measured with an aspirin-sensitive laboratory test have been termed "aspirin resistant" but this somewhat misleading term should be strictly reserved for exceptional situations in which the drug is unable to hit its pharmacological target.[4] High on-aspirin platelet reactivity (HAPR) is a much more appropriate term to describe the high platelet reactivity status despite aspirin therapy in an individual patient. Moreover, it should be emphasized that a growing body of evidence shows a clear relationship between HAPR as measured with laboratory tests sensitive for platelet COX-1-inhibition and the occurrence of atherothrombotic events.[5-15]

In the last couple of years, several platelet function tests have been developed, providing direct and indirect measurements of cyclooxygenase-1 (COX-1)-inhibition.[16] However, as yet, there is no consensus on the optimal method to assess the magnitude of on-aspirin platelet reactivity.[17] Therefore, the aim of present study is to assess which platelet function test specific for platelet COX-1-inhibition is best in predicting atherothrombotic events, including stent thrombosis, in patients undergoing an elective PCI with coronary stent implantation.

METHODS

Study Population

The POPular-study (*The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI-study)* was a prospective, observational study that included consecutive patients with established coronary artery disease scheduled for elective PCI with stent implantation. The entry and exclusion criteria were described in the original publication, describing platelet function tests specific for clopidogrel.[18] All patients were on dual antiplatelet therapy with clopidogrel and low-dose aspirin of 80-100 mg daily for at least 10 days. Compliance was verified by a detailed interview upon enrolment (self-reportage) as well as by pharmacy refill data.

Patients using concomitant medication known to affect platelet function other than clopidogrel and aspirin (i.e. NSAIDs, dipyramidole, upstream GP IIb/IIIa inhibitors), patients with a known platelet function disorder or a whole blood platelet count <150.000/ µL were excluded.

All coronary interventions were performed according to current guidelines and the choice of stent type and periprocedural use of glycoprotein (GP) IIb/IIIa inhibitors was left to the operator's discretion, but the latter were always administered after blood collection. Written informed consent was obtained before PCI. All data were prospectively collected and entered into a central database. Clinical follow-up was obtained by contacting all patients at 12 months and a double-check was performed on the basis of source documents obtained from medical records from the referring hospitals.

The study was conducted according to the principles of the Declaration of Helsinki and the laws and regulations applicable in the Netherlands. The local institutional review board (Verenigde Commissies Mensgebonden Onderzoek) approved the study.

Follow-up and endpoints

The primary endpoint was defined as a composite of all-cause death, non-fatal myocardial infarction (defined as the occurrence of ischemic symptoms and a spontaneous [i.e. not peri- or post-procedural] troponin T value or creatine kinase MB greater than the upper limit of normal), stent thrombosis (definite stent thrombosis according to the Academic Research Consortium criteria) and ischemic stroke (focal loss of neurologic function caused by an ischemic event). The primary safety endpoint was defined as major or minor bleeding according to the modified Thrombolysis In Myocardial Infarction (TIMI) Study Group criteria.[19] An independent committee, blinded for platelet function data, adjudicated all endpoints through review of source documents of medical records.

Blood sampling

Before heparinization, whole blood was drawn from the femoral or radial artery sheath. Blood samples were collected into 3.2% citrate Sarstedt tubes for light transmittance aggregometry (LTA) and the IMPACT-*R*. The VerifyNow® was performed using 3.2% citrate Greiner tubes, according to manufacturer's recommendation. For the PFA-100® System (Siemens Healthcare Diagnostics Products GmbH, Germany) 3.8% buffered citrated blood was used, according to the manufacturer's test protocol. Blood samples for whole blood count were drawn into tubes containing K_3-EDTA.

Platelet Function Measurements

The magnitude of on-aspirin platelet reactivity was quantified using four commercially available platelet function tests that claim to be sensitive for platelet COX-1-inhibtion: LTA using arachidonic acid (AA) as the agonist, the VerifyNow® Aspirin assay, the IMPACT-*R* assay (with AA pre-stimulation) and the Dade® PFA Collagen/Epinephrine Test Cartridge (COL/EPI). All platelet function measurements were performed between 30 minutes and 2 hours after blood collection.

Light Transmittance Aggregometry

LTA was performed in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and maximal platelet aggregation (%) was measured using AA in a final concentration of 0.5 mg/ml. In medical literature, the currently accepted cut-off value for AA-induced LTA to segregate patients with from those without HAPR is 20% aggregation.[5]

The VerifyNow® Aspirin-assay

The VerifyNow® system (Accumetrics, San Diego, USA) is a whole blood cartridge-based method

to determine the magnitude of AA-induced platelet agglutination (in a final concentration of 1mmol/L).[20]

After a citrated tube of whole blood is inserted into the cartridge, the platelets become activated by conversion of AA to TXA2 by COX-1. As a result, the activated platelets bind via GP IIb/IIIa-receptors to fibrinogen-coated beads and cause agglutination. Infrared-light transmittance through the chamber increases as the platelet-bead complexes fall out of the solution. The results are reported in aspirin reaction units (ARU). In medical literature, the currently accepted cut-off value for the VerifyNow® Aspirin-assay to segregate patients with from those without HAPR is 550 ARU.[13]

The IMPACT-R AA

The IMPACT-*R* device (DiaMed, Cresier, Switzerland) is based on the cone and plate(let) analyzer technology.[21] Citrated whole blood samples (130 µL) were, after pre-incubation with a concentration AA (0.32 µM) and a gentle mixture (10 RPM) for 1 minute, placed in a polystyrene well and subjected to a shear rate of $1800s^{-1}$ for 2 minutes using a Teflon Cone. When shear stress is applied, von Willebrand Factor and fibrinogen are instantly immobilized on the polystyrene surface, serving as a substrate for platelet adhesion and subsequent aggregation. The wells are washed and stained with May-Grünwald stain and analyzed with an inverted light microscope connected to an image analysis system. Platelet adhesion and aggregation on the surface were evaluated by examining the percentage of total area covered with platelets designated as surface coverage (SC).

Exposure to AA leads to the formation of micro-aggregates in patients in whom aspirin does not effectively inhibit platelet function. These micro-aggregated platelets temporarily lose their adhesive properties. The percentage SC in the AA pre-stimulated aliquots is therefore inversely correlated with the magnitude of AA-induced platelet activation. In medical literature, the currently accepted cut-off value for the IMPACT-R AA to segregate patients with from those without HAPR is 2.5 % SC.[21]

PFA-100® System

The PFA-100® System (Siemens Healthcare Diagnostics Products GmbH, Germany),

measures platelet function, in particular adhesion and aggregation, in whole blood under high shear conditions ($5000s^{-1}$). The time needed to form a platelet plug occluding the aperture cut into a collagen/epinephrine (COL/EPI)-coated membrane was determined and reported as closure time (CT) in seconds. The closure time inversely reflects the magnitude of platelet reactivity. In medical literature, the current accepted cut-off value for the PFA COL/EPI to segregate patients with from those without HAPR is 193 seconds.[7]

Statistical analysis

Continuous variables are presented as mean (SD). Categorical data are reported as frequencies (percentages). Categorical variables were compared using the chi-square test. Normally distributed

continuous variables were compared with a two-sided unpaired t test.

To evaluate a platelet function assay's capability to discriminate between patients with and without primary endpoint at one-year follow-up, a receiver-operator characteristic (ROC) curve analysis was calculated for each test. The optimal cut-off level was calculated by determining the smallest distance between the ROC-curve and the upper left corner of the graph. Patients above this optimal cut-off level were considered to exhibit HAPR. Survival analysis for patients with and without HAPR according to the ROC of the specific test, were performed using the Kaplan-Meier method, and the differences between groups were assessed by the log-rank test. The measure of effect was the Odds Ratio (OR) and estimated from a logistic regression analysis. To correct for over fit of the ROC-curve derived cut-off value, a statistical resampling methodology (bootstrapping) was performed to assess the mean and distribution of the cut-off values derived from the area under the corresponding ROC curve (area under the curve[AUC]). A total of 1000 replicates of each data set were created by resampling with replacement; each resampled dataset consisted of the same size as the original. For each platelet function test, the ROC curve was generated and the AUC was computed and subsequently the cut-off value, along with the 95%-confidence interval. Furthermore, the cut-off levels for the four tests were derived from medical literature and were applied to our clinical outcome data.

Logistic regression modelling was used to identify independent correlates of the primary endpoint. The model included on-treatment platelet reactivity according to the various tests as a categorical variable (HAPR vs patients without HAPR using the cut-off defined with the ROC-analysis) and multiple potential confounders (classic cardiovascular risk factors, renal failure, left ventricular ejection fraction <45%, total stent length, number of lesions treated, amount of stents implanted, bifurcation lesions, co-medication [including use of clopidogrel loading dose, coumadins, proton pump inhibitors, calcium channel blockers, statins or GP IIb/IIIa inhibitors], laboratory parameters [hemoglobin, platelet count and mean platelet volume], left anterior descendens coronary artery (LAD) or graft-stenting). All univariate variables with a p-value <0.10 were included in multivariable analysis. Whether a variable had additional contribution to a logistic regression model without that variable was tested with the likelihood-ratio test. The Hosmer-Le Cessie goodness-of-fit test was performed to assess the adequacy of the model. All statistical analyses were performed with R (version 2.9,http://www.r-project.org) and a two-tailed p-value of <0.05 was considered significant.

RESULTS

A total of 1069 consecutive patients were enrolled, of whom 951 were on aspirin >10 days.

Owing to irregularities in platelet assay supply, as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient. Furthermore, halfway through the POPular-study the VerifyNow® Aspirin cartridge was included. As a consequence, AA-induced LTA was performed in a total of 925 patients; the VerifyNow® Aspirin cartridge in 422 patients; IMPACT-*R* in 791 patients and the PFA COL/EPI in 719 patients.

Baseline characteristics of the cohort are depicted in **Table 1**. Baseline characteristics of the subpopulations according to the tests performed are summarized in **Appendix Table 1**, demonstrating that the subpopulations tested were well balanced (all p-values>0.55).
Clinical outcome at 12 months was available for 949 (99.9%) of the patients. During one-year follow-up a total of 16 (1.7 %) patients died, 54 (5.7%) patients had non-fatal acute myocardial infarction, 9 (0.9%) patients presented with definite stent thrombosis and 11 (1.2%) patients suffered from non-fatal ischemic stroke. A total of 39 (4.1%) patients presented with bleeding; 23 (2.4%) TIMI-major and 19 (1.9%) TIMI-minor bleedings.

Receiver-Operating Characteristic Curve Analysis

Receiver operator characteristic curve (ROC) analysis demonstrated that only aggregation based tests such as AA-induced LTA and the VerifyNow® Aspirin-cartridge assay were able to discriminate between patients with and without primary endpoint at 1-year follow-up (Appendix **Figure 1**). The estimate of area under the curve (AUC) obtained by resampling was almost identical to the parametric estimate and the bias of the analysis varied between 0.6% and 3.0% (**Table 2**). In contrast, neither the shear stress based test IMPACT-*R* with AA pre-stimulation, nor the PFA COL/EPI Test were able to distinguish between patients with and without post-procedural events. **Table 2** displays the AUC and optimal cut-off value for every test, including the estimated cut-off value after bootstrap analysis.

Baseline characteristics for every test, for patients with and without HAPR, are depicted in **Appendix Table2**. Patients exhibiting HAPR according to AA-induced LTA were significantly older, the proportion of females was higher, the frequency of diabetes mellitus was higher, had a lower platelet count and were less often treated with statins. In patients with HAPR as measured by the VerifyNow® Aspirin-cartridge the proportion of diabetic patients and patients with hypertension was higher. Patients with HAPR according to the IMPACT-R AA were more likely to be female or be a non-smoker, had a higher BMI, suffered less often from hypertension and were treated more often with ACE-inhibition. Patients who exhibited HAPR as defined by PFA-100 COL/EPI were older, were more often female, had a lower hemoglobin, more often suffered from diabetes or renal failure, had more often a history of prior myocardial infarction and an impaired ejection fraction.
Logistic regression modelling was used to determine independent predictors for the primary endpoint. The model included on-treatment platelet reactivity according to the various tests as a categorical variable (HAPR vs patients without HAPR using the cut-off defined with the ROC-analysis) and multiple potential confounders. Clinical factors independently predicting the 1-year primary endpoint were age (calculated for an increase of 10 years (OR = 1.30; 95%-CI: 0.99-1.06), hypertension (OR = 2.33; 95%-CI: 1.04-5.19), and a left ventricular ejection fraction < 45% (OR = 1.82; 95%-CI: 0.91-3.63). Procedural factors independently predicting the primary endpoint were graft-stenting (OR = 3.31; 95%-CI: 0.98-11.21) and stenting of a bifurcation lesion (OR =4.77; 95%-CI:1.28-17.85). The remaining variables included for multivariate analysis were not found to be independent

Table 1 Baseline characteristics total population

Clinical parameters	Total population
Age	64 ± 10.6
Gender (male)	717/951 (75.4%)
Hypertension	737/951 (77.5%)
Hypercholesterolemia	769/951 (80.9%)
Diabetes Mellitus	175/951 (18.4%)
Family history	580/951 (61.0%)
Current smoking	107/951 (11.3%)
LVEF<45%	133/951 (14.0%)
Renal insufficiency	72/951 (7.6%)
Prior myocardial infarction	519/951 (54.6%)
Medication	
Loading dose clopidogrel	489/951 (51.4%)
PPI	270/951 (28.4%)
Coumadins	24/951 (2.5%)
Laboratory Parameters	
Platelet count (x10^9)	273.4 ± 78.8
WBC (x10^9)	7.7 ± 2.3
Hemoglobin (g/dL)	13.8 ± 3.5
Procedural Parameters	
Mean no.of stents implanted	1.58
Minimal stent diameter (mm)	3.1 ± 0.8
Total stent length (mm)	28.3 ± 17.1
Bifurcation lesion	32/951 (3.4%)
Drug eluting stent	604/946 (63.8%)
LAD	450/951 (47.3%)

LVEF = left ventricular ejection fraction; PPI =proton pump inhibitors, CCB = calcium channel blockers; WBC = white bloodcell count, LAD = Left Anterior Descending Artery

Definitions

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.
Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.
Diabetes mellitus: According to the World Health Organization criteria
Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).
Renal insufficiency: Serum creatinine > 120 µmol/L

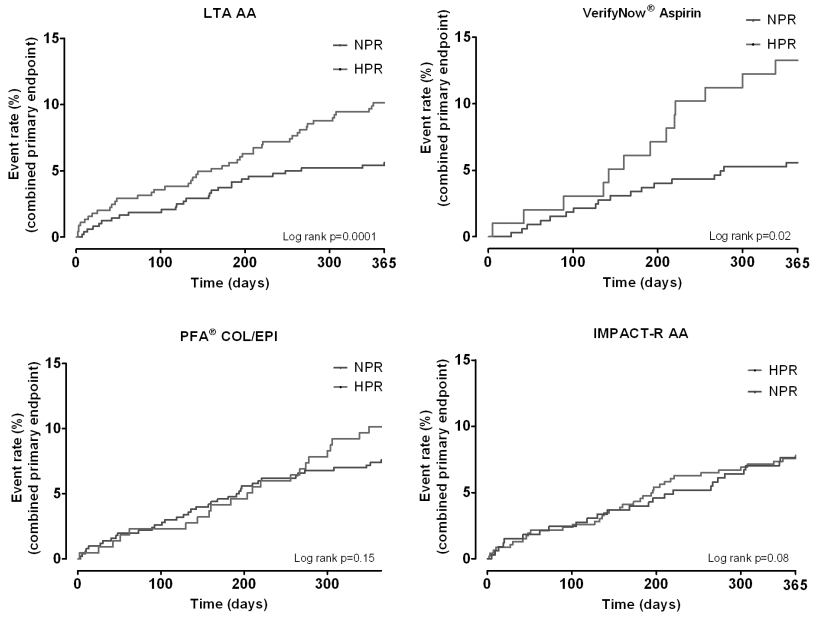

Figure 1: Kaplan-Meier Analysis

Kaplan Meier analysis for the event rate from the combined primary endpoint in patients with and without HPR as measured by the multiple platelet function assays.

HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity

correlates of the primary endpoint (p>0.10) and were not included in the model.

The addition of HAPR to this statistical model revealed that HAPR as measured with AA-induced LTA and the VerifyNow® Aspirin-cartridge significantly improved the AUC (AUC=0.73, p=0.03 and AUC=0.78, p=0.02 respectively). Likewise, the likelihood-ratio test demonstrated that HAPR according to these tests had additional contribution to the model (**Table 3**). The goodness-of-fit test demonstrated that the predicting model was adequate (except for the PFA COL/EPI, p=0.05, all p-values>0.50). On the contrary, the AUC did not improve when HAPR as measured with IMPACT-*R* or the PFA COL/EPI was added to the model.

Relationship between high on-aspirin platelet reactivity and clinical outcome

The primary endpoint occurred more frequently in patients with HAPR compared to patients without HAPR when platelet function was assessed by LTA (10.1% vs 6.0%, p=0.02) and the VerifyNow® Aspirin-assay (13.3% vs 5.9%, p=0.015) using the cut-off levels of the ROC-analysis. One-year follow-up for patients with and without HAPR according to each platelet function test is depicted in **Table 4**. The combined endpoint occurred significantly more often in patients with HAPR when measured with AA-induced LTA and the VerifyNow® Aspirin-cartridge as compared to patients without HAPR, whereas no significant association was detected when platelet function was assessed by the IMPACT-*R* or by the PFA COL/EPI **(Figure 1**).

Table 2: Area under the curve

	AUC	95% CI	Cut-Off	Cut-Off after bootstrapping	Bias	Sensitivity	95% CI	Specificity	95%CI	NPV	PPV
LTA AA	0.58	0.52-0.64	20%	19.6%	2.0%	64.0	52.7-73.9	52.6	49.2-55.9	94.0%	10.3%
VerifyNow ARU	0.62	0.52-0.71	454 ARU	440.6 ARU	3.0%	43.8	28.3-60.7	77.8	73.4-81.6	94.2%	13.3%
PFA Collagen/Epinephrine	0.53	0.48-0.58	299 seconds	296.6 seconds	0.8%	38.7	27.6-51.1	70.3	66.7-73.7	92.4%	11.0%
IMPACT-R AA	0.51	0.48-0.61	7.2 %SC	7.16 %SC	0.6%	40.6	29.5-52.9	58.7	55.1-62.2	92.1%	8.1%

AUC and optimal cut-off values for each test

AUC = Area under the curve CI = confidence interval NPV = negative predictive value PPV = positive predictive value LTA=light transmittance aggregometry AA= arachidonic acid ARU = Aspirin reaction units SC= surface coverage HPR=high on-treatment platelet reactivity

Table 3: AUC of different backward regression models for the prediction of the primary end point at one-yearfollow-up

	AUC	p-value for addition§
Model 1: Classical cardiovascular risk factors†	0.66	
Model 2: Model 1 + procedural risk factors‡	0.72	0.0001
Model 3: Model 2 + residual platelet reactivity		
- LTA AA	0.73	0.03
-VerifyNow ARU	0.78	0.02
- PFA-100 COL/EPI	0.72	0.44
-IMPACT-R AA	0.72	0.94

† Age, hypertension, LVEF (left ventricular ejection fraction) < 45%,
‡ Graft-stenting, stenting of a bifurcation lesion.
§ Likelihood Ratio Test for additional value of HPR as measured with multiple platelet function test.

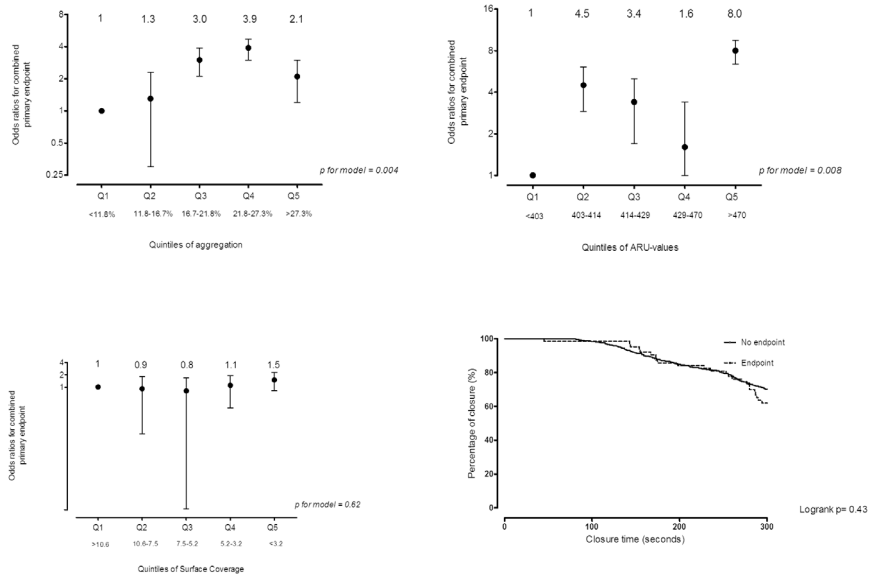

Figure 2: Odds Ratios for the primary endpoint
Odds Ratios for the combined primary endpoint by quintiles of on-treatment platelet reactivity according to multiple platelet function assays. Error bars indicate 95%confidence intervals. Cumulative Kaplan Meier time-to-aperture-closure plot in patients with and without the combined primary endpoint according to the PFA-100® System. Q = quintile

The occurrence of the primary end point was depicted in quintiles according to on-treatment platelet reactivity (**Figure 2**). Patients in the higher quintiles according to the AA-induced LTA (p=0.004) and the VerifyNow® Aspirin-assay (p=0.008) were at significantly higher risk for the primary end point. In contrast, no significant difference in the occurrence of the primary endpoint was observed between

Table 4: Clinical Outcome

	LTA AA			
	NPR (n=481)	HPR (n=444)	OR (95 CI)	p-value
	< 20% aggregation	> 20 % aggregation		
Death, MI, ST, stroke	29 (6.0%)	45 (10.1%)	1.76 (1.08-2.86)	0.020
Death	4 (0.8%)	11 (2.5%)	3.03 (0.96-9.58)	0.048
MI	21 (4.4%)	31 (7.0%)	1.64 (0.93-2.91)	0.08
ST	3 (0.6%)	6 (1.4%)	2.18 (0.54-8.78)	0.26
Stroke	6 (1.2%)	4 (0.9%)	0.72 (0.20-2.57)	0.61

	VerifyNow Aspirin			
	NPR (n=324)	HPR (n=98)	OR (95% CI)	p-value
	<454 ARU	>454 ARU		
Death, MI, ST, stroke	19 (5.9%)	13 (13.3%)	2.46 (1.17-5.17)	0.015
Death	5 (1.5%)	4 (4.1%)	2.71 (0.71-10.31)	0.13
MI	13 (4.0%)	6 (6.1%)	1.56 (0.58-4.22)	0.38
ST	1 (0.3%)	2 (2.0%)	6.73 (0.60-75.02)	0.07
Stroke	2 (0.6%)	3 (3.1%)	5.08 (0.84-30.87)	0.05

	PFA 100 COL/EPI			
	NPR (n=500)	HPR (n=219)	OR (95% CI)	p-value
	CT>299 seconds	CT <= 299 seconds		
Death, MI, ST, stroke	38 (7.6%)	24 (11.0%)	1.49 (0.88-2.56)	0.18
Death	7 (1.4%)	7 (3.2%)	2.33 (0.81-6.67)	0.19
MI	30 (6.0%)	14 (6.4%)	1.08 (0.56-2.04)	0.97
ST	3 (0.6%)	3 (1.4%)	2.33 (0.46-11.1)	0.55
Stroke	3 (0.6%)	4 (1.8%)	3.12 (0.68-14.3)	0.26

	IMPACT-R AA			
	NPR (n=327)	HPR (n=464)	OR (95% CI)	p-value
	SC>7.2%	SC<7.2%		
Death, MI, ST, stroke	25 (7.6%)	38 (8.2%)	1.08 (1.64-1.82)	0.91
Death	8 (2.4%)	6 (1.3%)	0.52 (0.18-1.52)	0.14
MI	13 (4.0%)	29 (6.2%)	1.61 (0.82-3.15)	0.16
ST	2 (0.6%)	5 (1.1%)	1.77 (0.34-9.18)	0.49
Stroke	4 (1.2%)	5 (1.1%)	0.88 (0.23-3.30)	0.85

HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity
MI = myocardial infarction, ST = Stent thrombosis

quintiles as measured with the IMPACT-*R* test. Since the PFA-100® System confines detection of a closure time to a 300-s window, the results of the PFA-cartridge are depicted as time to aperture closure Kaplan-Meier curves. Closure times as measured by the PFA COL/EPI were not significantly different between patients with and without a primary endpoint.

When literature derived cut-offs were applied to the dataset non of the tests were able to identify patients at higher risk for an atherothrombotic event, except for AA-induced LTA (ROC curve analysis derived cut-off is perfectly consistent with the one provided by medical literature (**Appendix Table 3**).

Relationship between platelet reactivity and bleeding

A second ROC-analysis demonstrated that the performed platelet function tests were unable to discriminate between patients with and without bleeding (all AUCs included 0.50 in the CI).

DISCUSSION

The principal finding of the present study is that high on-aspirin platelet reactivity as measured with aggregation-based tests specific for COX-1 inhibition by using arachidonic acid as the agonist is significantly associated with the occurrence of atherothrombotic events. In contrast, the shear stress based tests IMPACT-*R* and the PFA-100 COL/EPI were not able to identify patients at risk for adverse clinical outcome.

Although previous observational studies have already demonstrated the relationship between high on-aspirin platelet reactivity and adverse clinical outcome, these studies were limited by small sample size, the use of clinically non-validated cut-off levels and the availability of only one test per study.[5-15]

The present study, which is substantially larger than previous studies, was designed to assess the predictive accuracy of four different platelet function tests identifying patients at higher risk for atherothrombotic events after PCI.

Among the four tests, the VerifyNow® Aspirin-assay clearly showed the best predictive value for the occurrence of adverse events. Using ROC curve analysis on clinical outcome data, we were able to identify an optimal cut-off of 454 ARU to segregate patients with and without HAPR. This cut-off level is substantially lower than the currently accepted cut-off value of 550 ARU.[3,13,22] However, the commonly used cut-off of 550 ARU is questioned[23,24] since it is not identified by ROC curve analysis and even more important, it has been determined with a previous design of the VerifyNow® Aspirin-assay that used cationic propyl gallate instead of arachidonic acid as agonist. The available evidence linking HAPR as measured with the VerifyNow® Aspirin-assay and clinical outcome were performed using propyl gallate [13] and our study is the first that shows the relationship using AA as the agonist. Remarkably, the VerifyNow aspirin assay loses its predictive ability using the cut-off value of 550 ARU. These findings suggest that a cut-off of 454 might be a more appropriate one to predict clinical outcome using the current design of this cartridge.

In line with previous studies[5,8,25] light transmittance aggregometry is also able to discriminate

between patients with and without atherothrombotic event. Our ROC-curve derived cut-off of 20% AA-induced aggregation is perfectly consistent with the one derived from literature.[5] However, the predictive accuracy of light transmittance aggregometry is only moderate and the test might not be suitable for routine use in clinical practice because of some major limitations including poor reproducibility, long sample processing time, labour intensiveness and the need for specialized technicians.[16]

The present study is the first to investigate the association between HAPR according to the IMPACT-R AA and adverse clinical outcome. The cut-off value determined by ROC-curve analysis (7.2%) differs from the cut-off value as recommended in literature (2.5%).[21] The IMPACT-R was not able to segregate patients with and without HAPR or to identify patients at higher risk of atherothrombotic events, neither using our cut-off nor the one derived from literature.

The PFA COL/EPI was also unable to discriminate between patients with and without primary endpoint. Contrasting results have been reported concerning the predictive accuracy of the PFA COL/EPI cartridge. Some studies demonstrated a two to five fold[9,26,27] higher risk in aspirin-treated patients with a shorter closure time, using either a cut-off value of 193 seconds or 300 seconds, whereas the largest study thus far demonstrated no association at all between high on-aspirin platelet reactivity according to the PFA COL/EPI and adverse clinical outcome.[7]

In accordance with earlier investigations, the prevalence of high on-treatment platelet reactivity is highly dependent on the type of platelet function assay used.[3] In addition, the platelet function tests used in the POPular-study are not equally predictive in identifying patients at higher risk of atherothrombotic events. The addition of high on-aspirin platelet reactivity according to the aggregation based COX-1-inhibition sensitive tests to a model that includes both classical and procedural risk factors moderately but significantly enhanced the predictability of this model. The VerifyNow® showed the largest increase in predictive value of all tests performed in this cohort (AUC=0.78) and therefore should be considered the best platelet function test to identify high-risk patients. Until now, no data are available concerning the clinical effectiveness of tailoring aspirin therapy based on the results of platelet function testing. Therefore, the correct treatment-if any-of high on-treatment platelet reactivity remains unknown.[17]

Some limitations merit careful consideration. First, the lack of data on serum thromboxane-B2, which is, being the stable metabolite of TXA2, considered the most specific measurement of platelet COX-1 activity, might have given more clarity in the determination of HAPR. Second, not all platelet function tests were performed in every patient. Third, the absence of an association between the magnitude of platelet reactivity and bleeding complications should be interpreted with care, since the present analysis was not powered to identify patients at higher risk of bleeding.

In conclusion, this parallel evaluation of platelet function tests in their ability to predict clinical outcome demonstrates that the VerifyNow® Aspirin-assay is best in identifying patients undergoing elective PCI who are at higher risk for atherothrombotic events. In contrast, none of the platelet function tests are able identifying patients at higher risk of bleeding. Since the adequate treatment

of high on-aspirin platelet reactivity is unknown, the routine use of platelet function testing in clinical practice is not recommended.

Appendix Table 1

	LTA AA n=925	VerifyNow ARU n=422	IMPACT-R AA n=791	PFA COL/EPI n=719
Clinical parameters				
Age (yrs)	64 ± 10.6	64 ± 11.2	64 ± 10.7	63 ± 10.5
BMI (kg/m2)	27.3 ± 3.9	27.2 ± 4.2	27.3 ± 3.9	27.3 ± 3.9
Gender (male)	696/925 (75.2%)	309/422 (73.2%)	596/791 (75.3%)	530/719 (73.7%)
Hypertension	721/925 (77.9%)	305/422 (72.3%)	619/791 (78.3%)	553/719 (76.9%)
Hypercholesterolemia	745/925 (80.5%)	330/422 (78.2%)	644/791 (81.4%)	581/719 (80.8%)
Diabetes Mellitus	169/925 (18.3%)	75/422 (17.8%)	140/791 (17.7%)	130/719 (18.1%)
Family History	564/925 (61.0%)	236/422 (55.9%)	476/791 (60.2%)	568/719 (61.3%)
Current smoking	102/925 (11.0%)	42/422 (10.0%)	83/791 (10.0%)	74/719 (10.3%)
LVEF < 45%	130/925 (14.1%)	54/422 (12.8%)	103/791 (13.0%)	92/719 (12.8%)
Renal insufficiency	71/925 (7.74%)	35/422 (8.3%)	57/791 (7.2%)	51/719 (7.1%)
Prior myocardial infarction	507/925 (54.8%)	243/422 (57.6%)	436/791 (55.1%)	407/719 (56.6%)
Prior PCI	301/925 (32.5%)	124/422 (29.4%)	251/791 (31.7%)	213/719 (29.6%)
Prior CABG	92/925 (9.9%)	54/422 (12.8%)	83/791 (10.5%)	75/719 (10.4%)
Medication				
Loading dose clopidogrel	473/925 (51.1%)	352/422 (49.0%)	202/791 (47.9%)	473/719 (51.0%)
Statin	746/925 (80.6%)	329/422 (78.0%)	647/791 (81.8%)	575/719 (80.0%)
Beta-blocker	711/925 (76.9%)	324/422 (76.8%)	6177/791 (78.0%)	555/719 (77.2%)
ACE-inhibitor	341/925 (36.9%)	151/422 (35.8%)	288/791 (36.4%)	268/719 (37.3%)
PPI	268/925 (29.0%)	128/422 (30.3%)	216/791 (27.3%)	205/719 (28.5%)
CCB	357/925 (38.6%)	149/422 (35.3%)	305/791 (38.4%)	274/719 (38.3%)
Oral antidiabetics	61/925 (6.6%)	19/422 (4.5%)	48/791 (6.1%)	46/719 (6.4%)
Coumadins	24/925 (2.6%)	11/422 (2.6%)	14/791 (1.8%)	14/719 (1.9%)
Laboratory Parameters				
Platelet count (x10^9)	273.5 ± 79.3	267.8 ± 78.3	274.5 ± 78.7	274.2 ± 79.9
WBC (x10^9)	7.7 ± 2.3	7.7 ± 2.4	7.7 ± 2.2	7.7 ± 2.3
Hemoglobin (g/dL)	13.8 ± 3.5	14.0 ± 5.0	13.8 ± 3.9	13.8 ± 3.9
Procedural Parameters				
Mean no.of stents implanted	1.56	1.54	1.57	1.55
Mean no. of lesions treated	1.39	1.32	1.38	1.34
Minimal Stent diameter (mm)	3.1 ± 0.9	3.1 ± 0.9	3.1 ± 0.9	3.1 ± 0.9
Total Stent length (mm)	28.3 ± 17.1	27.2 ± 15.7	28.3 ± 16.8	27.7 ± 16.5
Bifurcation lesion	30/925 (3.2%)	10/422 (2.4%)	24/791 (2.4%)	19/719 (2.6%)
Drug eluting stent	587/920 (63.8%)	270/422 (64.0%)	505/786 (64.0%)	454/717 (63.3%)
LAD	437/925 (47.2%)	211/422 (50.0 %)	370/791 (50.0%)	348/719 (48.4%)
Graft	28/925 (3.0%)	14/422 (3.3%)	24//791 (3.3%)	20/719 (2.8%)

Appendix Table 1: Baseline characteristics of the subpopulations according to the available Platelet function measurements

LTA = light transmittance aggregometry; AA= arachidonic acid; BMI = Body Mass Index; LVEF = left ventricular ejection fraction; PCI = percutaneous coronary intervention; CABG = coronary artery bypass grafting; PPI =proton pump inhibitors; CCB = calcium channel blockers; WBC = white bloodcell count; LAD = Left Anterior Descending Artery

Definitions as in Table 1:

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.
Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.
Diabetes mellitus: According to the World Health Organization criteria [28]
Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).

Renal insufficiency: Serum creatinine > 120 µmol/L

Appendix Table 2

	LTA AA			VerifyNow Aspirin		
Clinical parameters	NPR (n=481)	HPR (n=444)	p-value	NPR (n=324)	HPR (n=98)	p-value
Age (yrs)	61 ± 10.0	67 ± 10.3	<0.001	64 ± 11.1	65 ± 11.5	0.26
BMI (kg/m2)	27.5 ± 3.8	270 ± 4.0	0.06	27.0 ± 4.0	27.6 ± 4.8	0.28
Gender (male)	388/481 (80.7%)	308/444 (69.4%)	<0.001	237/324 (73.1%)	72/98 (73.5%)	0.95
Hypertension	382/481 (79.4%)	339/444 (76.4%)	0.26	226/324 (69.8%)	79/98 (80.6%)	0.04
Hypercholesterolemia	391/481 (81.3%)	354/444 (79.7%)	0.55	258/324 (79.6%)	72/98 (73.5%)	0.20
Diabetes Mellitus	76/481 (15.8%)	93/444 (20.9%)	0.04	47/324 (14.5%)	28/98 (28.6%)	0.001
Family History	298/481 (62.0%)	266/444 (59.9%)	0.52	187/324 (57.7%)	49/98 (50.0%)	0.18
Current smoking	60/481 (12.5%)	42/444 (9.5%)	0.14	33/324 (10.2%)	9/98 (9.2%)	0.77
LVEF < 45%	59/481 (12.3%)	71/444 (16.0%)	0.10	44/324 (13.6%)	10/98 (10.2%)	0.38
Renal insufficiency	30/481 (6.2%)	41/444 (9.2%)	0.09	25/324 (7.7%)	10/98 (10.2%)	0.43
Prior MI	274/481 (57.0%)	233/444 (52.5%)	0.17	187/324 (57.7%)	56/98 (57.1%)	0.92
Prior PCI	158/481 (32.8%)	143/444 (32.2%)	0.84	95/324 (29.3%)	29/98 (29.6%)	0.96
Prior CABG	46/481 (9.6%)	46/444 (10.4%)	0.69	44/324 (13.6%)	10/98 (10.2%)	0.38
Medication						
Aspirin	481/481 (100%)	444/444 (100%)	1.00	324/324 (100%)	98/98 (100%)	1.00
Loading dose clopidogrel	253/481 (52.6%)	220/444 (49.6%)	0.35	153/324 (47.2%)	49/98 (50.0%)	0.63
Statin	400/481 (83.2%)	346/444 (77.9%)	0.04	256/324 (79.0%)	73/98 (74.5%)	0.34
Beta-blocker	361/481 (75.1%)	350/444 (78.8%)	0.17	247/324 (76.2%)	77/98 (78.6%)	0.63
ACE-inhibitor	165/481 (34.3%)	176/444 (39.6%)	0.09	115/324 (35.5%)	36/98 (36.7%)	0.82
PPI	150/481 (31.2%)	118/444 (26.6%)	0.12	98/324 (30.2%)	30/98 (30.6%)	0.95
CCB	186/481 (38.7%)	171/444 (38.5%)	0.96	113/324 (34.9%)	36/98 (36.7%)	0.74
Oral antidiabetics	33/481 (6.9%)	28/444 (6.3%)	0.73	15/324 (4.6%)	4/98 (4.1%)	0.82
Coumadins	9/481 (1.9%)	15/444 (3.4%)	0.15	8/324 (2.5%)	3/98 (3.1%)	0.75

Laboratory Parameters						
Platelet count (x10^9)	281.5 ± 81.9	265.9 ± 75.5	0.004	271.1 ± 75.8	235.6 ± 84.7	0.06
WBC (x10^9)	7.7 ± 2.4	7.6 ± 2.2	0.60	7.7 ± 2.3	7.8 ± 2.8	0.69
Haemoglobin (g/dL)	14.0 ± 1.5	13.7 ± 5.0	0.21	13.8 ± 10.0	14.3 ± 10.0	0.56
Procedural Parameters						
Mean no.of stents implanted	1.55	1.58	0.81	1.38	1.29	0.47
Mean no.of lesions treated	1.35	1.41	0.05	2.05	2.20	0.70
Minimal Stent diameter (mm)	3.1 ± 1.1	3.0 ± 0.4	0.09	3.0 ± 0.4	3.2 ± 2.1	0.33
Total Stent length (mm)	28.5 ± 17.2	28.2 ± 17.1	0.83	27.5 ± 15.6	26.2 ± 15.9	0.49
Bifurcation lesion	12/481 (2.5%)	18/444 (4.1%)	0.18	8/324 (2.5%)	2/98 (2.0%)	0.81
Drug eluting stent	312/480 (65.0%)	275/440 (62.5%)	0.48	307/324 (5.2%)	88/98 (89.8%)	0.21
LAD	217/481 (45.1%)	220/444 (49.6%)	0.18	164/324 (50.6%)	47/98 (48.0%)	0.65
Graft	16/481 (3.3%)	12/444 (2.7%)	0.58	11/324 (3.4%)	39/98 (3.1%)	0.87

	IMPACT-R AA			**PFA COL/EPI**		
Clinical parameters	NPR (n=327)	HPR (n=464)	p-value	NPR (n=500)	HPR (n=219)	p-value
Age (yrs)	63 ± 10.6	64 ± 10.7	0.40	63 ± 10.3	65 ± 10.8	0.0009
BMI (kg/m2)	26.9 ± 3.6	27.5 ± 4.1	0.04	27.1 ± 4.1	27.6 ± 3.7	0.19
Gender (male)	258/327 (78.9%)	338/464 (72.8%)	0.01	381/500 (76.2%)	149/219 (68.0%)	0.028
Hypertension	239/327 (73.1%)	380/464 (81.9%)	0.02	378/500 (75.6%)	175/219 (79.0%)	0.24
Hypercholesterolemia	260/327 (79.5%)	384/464 (82.8%)	0.69	407/500 (81.4%)	174/219 (79.5%)	0.61
Diabetes Mellitus	48/327 (14.7%)	92/464 (19.8%)	0.09	77/500 (15.4%)	53/219 (24.2%)	0.007
Family History	201/327 (61.5%)	275/464 (59.3%)	0.29	303/500 (60.6%)	133/219 (60.7%)	0.96
Current smoking	43/327 (13.1%)	40/464 (8.6%)	0.03	55/500 (11.0%)	19/219 (8.7%)	0.42
LVEF < 45%	40/327 (12.2%)	63/464 (13.6%)	0.68	55/500 (11.0%)	37/219 (16.9%)	0.04
Renal insufficiency	21/327 (6.4%)	36/464 (7.8%)	0.54	28/500 (5.6%)	23/219 (10.5%)	0.03
Prior MI	184/327 (56.3%)	252/464 (54.3%)	0.34	298/500 (59.6%)	109/219 (49.8%)	0.02
Prior PCI	103/327 (31.5%)	148/464 (31.9%)	0.90	147/500 (29.4%)	66/219 (30.1%)	0.91
Prior CABG	34/327 (10.4%)	49/464 (10.6%)	0.96	47/500 (9.4%)	28/219 (12.8%)	0.22

Medication						
Aspirin	327/327 (100%)	464/464 (100%)	1.00	500/500 (100%)	219/219 (100%)	1.00
Loading dose clopidogrel	155/327 (47.4%)	251/464 (54.1%)	0.14	248/500 (49.6%)	104/219 (47.5%)	0.66
Statin	255/327 (80.0%)	392/464 (84.5%)	0.12	397/500 (79.4%)	178/219 (81.3%)	0.63
Beta-blocker	259/327 (79.2%)	358/464 (77.2%)	0.17	384/500 (76.8%)	171/219 (78.1%)	0.78
ACE-inhibitor	99/327 (30.3%)	189/464 (40.7%)	0.006	183/500(36.6%)	85/219 (38.8%)	0.63
PPI	88/327 (26.9%)	128/464 (27.6%)	0.99	143/500 (28.6%)	62/219 (28.3%)	0.99
CCB	128/327 (39.1%)	177/464 (38.1%)	0.57	198/500 (39.6%)	76/219 (34.7%)	0.25
Oral antidiabetics	18/327 (5.5%)	30/464 (6.5%)	0.64	29/500 (5.8%)	17/219 (7.8%)	0.41
Coumadins	7/327 (2.1%)	7/464 (1.5%)	0.48	7/500 (1.4%)	7/219 (3.2%)	0.19
Laboratory Parameters						
Platelet count (x10^9)	274.6 ±79.4	274.5 ± 78.4	0.99	273.2 ± 79.7	276.7 ± 80.5	0.60
WBC (x10^9)	7.8 ± 2.2	7.7 ± 2.1	0.64	7.7 ± 2.2	7.6 ± 2.3	0.56
Haemoglobin (g/dL)	14.0 ± 1.5	13.7 ± 4.8	0.13	14.2 ± 4.5	13.3 ± 1.6	0.0002
Procedural Parameters						
Mean no.of stents implanted	1.60	1.54	0.28	1.55	1.54	0.56
Mean no.of lesions treated	1.35	1.40	0.23	1.34	1.33	0.63
Minimal Stent diameter (mm)	29.4 ± 16.9	27.5 ± 16.7	0.12	3.0 ± 0.5	3.1 ± 1.4	0.30
Total Stent length (mm)	3.1 ± 1.2	3.1 ± 0.6	0.97	27.9 ± 15.9	27.2 ± 17.9	0.57
Bifurcation lesion	13/327 (3.9%)	11/464 (2.4%)	0.17	14/500 (2.8%)	5/219 (2.3%)	0.88
Drug eluting stent	207/325 (63.7%)	298/461 (64.6%)	0.96	308/498 (61.8%)	136/219 (62.1%)	0.98
LAD	148/327 (45.3%)	222/464 (47.8%)	0.70	244/500 (48.8%)	104/219 (47.5%)	0.81
Graft	7/327 (2.1%)	17/464 (3.7%)	0.25	10/500 (2.0%)	10/219 (4.6%)	0.09

Appendix Table 2: Baseline characteristics of the study population according to the magnitude of platelet reactivity

Baseline characteristics of the subpopulations according to the available platelet function measurements, divided into two group, according to HPR and NPR HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity

Further abbreviations as in Appendix Table 1.

Definitions as in Table 1:

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.

Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.

Diabetes mellitus: According to the World Health Organization criteria

Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).

Renal insufficiency: Serum creatinine > 120 µmol/L

Appendix Table 3: Clinical Outcome using cut-off values derived from literature

	LTA AA NPR (n=481) < 20% aggregation	HPR (n=444) > 20 % aggregation	OR (95 CI)	p-value
Death, MI, ST, stroke	29 (6.0%)	45 (10.1%)	1.76 (1.08-2.86)	0.02
Death	4 (0.8%)	11 (2.5%)	3.03 (0.96-9.58)	0.048
MI	21 (4.4%)	31 (7.0%)	1.64 (0.93-2.91)	0.08
ST	3 (0.6%)	6 (1.4%)	2.18 (0.54-8.78)	0.26
Stroke	6 (1.2%)	4 (0.9%)	0.72 (0.20-2.57)	0.61

	VerifyNow Aspirin NPR (n=407) <550 ARU	HPR (n=15) >550 ARU	OR (95% CI)	p-value
Death, MI, ST, stroke	30 (7.4%)	2 (13.3%)	1.93 (0.42-8.97)	0.39
Death	8 (2.0%)	1 (6.7%)	3.56 (0.42-30.47)	0.22
MI	18 (4.4%)	1 (6.7%)	1.54 (0.19-12.39)	0.68
ST	2 (0.5%)	1 (6.7%)	14.46 (1.24-169.12)	0.005
Stroke	5 (1.2%)	0 (0%)	0.01 (0-	0.67

	PFA 100 COL/EPI NPR (n=616) CT>193 seconds	HPR (n=103) CT <193 seconds	OR (95% CI)	p-value
Death, MI, ST, stroke	53 (8.6%)	9 (8.7%)	0.98 (0.47-2.06)	0.96
Death	13 (2.1%)	1 (1.0%)	2.20 (0.28-16.99)	0.44
MI	39 (6.3%)	5 (4.9%)	1.32 (0.51-3.44)	0.56
ST	5 (0.8%)	1 (1.0%)	0.83 (0.10-7.22)	0.87
Stroke	3 (0.5%)	4 (3.9%)	0.12 (0.03-0.55)	0.001

	IMPACT-R AA NPR (n=681) SC>2.5 %	HPR (n=110) SC<2.5 %	OR (95% CI)	p-value
Death, MI, ST, stroke	51 (7.5%)	12 (10.9%)	1.51 (0.78-2.94)	0.22
Death	10 (1.5%)	4 (3.6%)	2.53 (0.78-8.22)	0.11
MI	35 (5.1%)	7 (6.4%)	1.25 (0.54-2.90)	0.60
ST	6 (0.9%)	1 (0.9%)	1.03 (0.12-8.66)	0.98
Stroke	8 (1.2%)	1 (0.9%)	0.77 (0.10-6.23)	0.81

HPR = high on-treatment platelet reactivity, NPR = normal on-treatment platelet reactivity, MI = myocardial infarction, ST = Stent thrombosis.

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Chapter 5

High on-treatment platelet reactivity to both aspirin and clopidogrel is associated with the highest risk of adverse events following percutaneous coronary intervention

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ABSTRACT

Aim: High on-clopidogrel platelet reactivity (HCPR) and high on-aspirin platelet reactivity (HAPR) are associated with atherothrombotic events following coronary stenting. There are, however, few data concerning high on-treatment platelet reactivity to both aspirin and clopidogrel simultaneously. The aim of the present study was to determine the incidence of dual high on-treatment platelet reactivity (DAPR) and its impact on clinical outcome.

Methods and results: On-treatment platelet reactivity was measured in parallel by ADP- and AA-induced light transmittance aggregometry (LTA) (n=921) and the point-off care VerifyNow®-System ((P2Y12 and Aspirin); n=422) in patients on dual antiplatelet therapy undergoing elective stent-implantation. HCPR and HAPR were established by receiver operator characteristic curve analysis. The primary endpoint was a composite of all-cause death, non-fatal acute myocardial infarction, stent thrombosis and ischemic stroke at one-year follow-up.

The incidence of DAPR varied between 14.7% and 26.9% depending on the platelet function test used. DAPR as assessed by LTA and the VerifyNow®-System was highly associated with adverse clinical outcome. At one-year follow-up the primary endpoint occurred more frequently in patients with isolated HCPR (11.7%), isolated HAPR (9.6%) or DAPR (10.7%) compared to patients without high on-treatment platelet reactivity (4.2%, all p-values<0.01) when platelet function was evaluated with LTA. Using the VerifyNow®-System, patients exhibiting DAPR had the highest risk for the primary endpoint (17.7% vs. 4.1% in patients without high on-treatment platelet reactivity, p=0.001).

Conclusions: In patients undergoing elective PCI dual high on-treatment platelet reactivity to aspirin and clopidogrel is present in 1 in 5 patients and is associated with a high risk for atherothrombotic events. Dual high on-treatment platelet reactivity measured by the point-of care VerifyNow®-System has a higher predictability for atherothrombotic events compared to light transmittance aggregometry.

INTRODUCTION

Dual antiplatelet therapy with aspirin and clopidogrel is pivotal to prevent atherothrombotic events in patients undergoing percutaneous coronary intervention (PCI) with stent-implantation.[1,2] However, the individual response to both drugs is heterogeneous and it has been demonstrated that high on-treatment platelet reactivity is associated with adverse outcome.[3-14] Furthermore, several studies have suggested that patients exhibiting high on-treatment platelet reactivity to both aspirin and clopidogrel simultaneously are at even higher risk of atherothrombotic events.[15-18] This is of utmost importance, since several studies have suggested the benefit of tailoring therapy in these patients.[19,20,9] The POPular- study (*The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI)* demonstrated that aggregation based tests were able to predict the occurrence of an adverse cardiovascular event in patients undergoing elective PCI with stent implantation.[10] The present sub-analysis aimed to explore the incidence of dual high on-treatment platelet reactivity (DAPR) and to assess whether patients exhibiting DAPR carry a higher risk of adverse events.

METHODS

A detailed description of the clinical characteristics of the patients and the entry and exclusion criteria of the POPular-study have been published previously.[10] The POPular was a head-to-head comparison between multiple platelet function assays, gauging the efficacy of clopidogrel, in their capability to predict atherothrombotic events. A recent sub-analysis studied the platelet function tests specific for aspirin.[21] In brief, the POPular-study was a prospective, observational study, enrolling consecutive patients with established coronary artery disease scheduled for elective PCI with stent-implantation. All patients received optimal clopidogrel treatment and all patients were on aspirin 80-100 mg daily ≥ 10 days, unless they were on long-term anticoagulation with coumarin derivates. Optimal pre-treatment clopidogrel regimens were defined as chronic maintenance therapy of 75 mg for >5 days or a clopidogrel loading dose of 300 mg at least 24 h before PCI or 600 mg at least 4 hours before PCI.

The patients on both aspirin and clopidogrel comprised the population in the present analysis. Compliance was verified by a detailed interview upon enrolment (self-reportage) as well as by pharmacy refill data. All interventions were performed according to current guidelines[22] and the choice of stent type and periprocedural use of glycoprotein (GP) IIb/IIIa inhibitors was left to the operator's discretion, but the latter were always administered after blood collection. Patients using concomitant medication known to affect platelet function other than aspirin (i.e. NSAIDs, dipyramidole, upstream GP IIb/IIIa inhibitors), patients with a known platelet function disorder or a whole blood platelet count <150.000/ µL were excluded. Clinical follow-up was obtained by contacting all patients at 12 months, which was verified on the basis of source documents from the medical records. The study was conducted according to the principles of the Declaration of Helsinki and the laws and regulations applicable in the Netherlands. The local institutional review board

approved the study.

Study design

The present study consisted of two sub-analyses; the primary comprised an analysis of the total population on both aspirin and clopidogrel (n=951) using light transmittance aggregometry (LTA) and the point-of-care VerifyNow®-System with the aim to establish whether patients exhibiting DAPR carry a higher risk of adverse events as compared to patients without high on-treatment platelet reactivity or high on-treatment platelet reactivity to either aspirin alone or clopidogrel alone. A secondary analysis was performed in those patients in whom both the VerifyNow®-System and LTA was performed (n=410), with the aim to compare the predictability of DAPR of both tests.

Follow-up and endpoints

The primary endpoint was defined as a composite of all-cause death, non-fatal myocardial infarction (defined as the occurrence of ischemic symptoms and a spontaneous [i.e. not peri- or post-procedural] troponin T value or creatine kinase MB greater than the upper limit of normal), stent thrombosis (definite stent thrombosis according to the Academic Research Consortium criteria)[23] and ischemic stroke (focal loss of neurologic function caused by an ischemic event).[24] An independent committee, blinded for platelet function data, adjudicated all endpoints through review of source documents of medical records.

Blood sampling

Before heparinization, whole blood was drawn from the femoral or radial artery sheath. Blood samples were collected into 3.2% Sarstedt® citrate tubes for light transmittance aggregometry (LTA). The VerifyNow®-System (Accumetrics, San Diego, USA) was performed using Greiner tubes, according to the manufacturer's recommendations. Blood samples for whole blood count were drawn into tubes containing K_3-EDTA.

Platelet Function Measurements

The magnitude of on-treatment platelet reactivity was quantified using the following platelet function tests; LTA using arachidonic acid (AA) and adenosine diphosphate (ADP) as the agonists and the VerifyNow®-System using the Aspirin and P2Y12 assays. All platelet function measurements were performed within 2 hours after blood collection.

Light Transmittance Aggregometry (LTA)

LTA was performed in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany) as previously described[25-29] Platelet-poor-plasma was set as 100% aggregation and maximal platelet aggregation (%) was measured using AA in a final concentration of 0.5 mg/ml and ADP in final concentrations of 5 and 20 µmol/L. The cut-off used

to identify patients with high on-clopidogrel platelet reactivity (HCPR) was 43% aggregation for 5 µmol/L ADP-induced LTA and 65% for 20 µmol/L ADP-induced aggregation.[10] The cut-off used to define high on-aspirin platelet reactivity (HAPR) was calculated by receiver-operator characteristic (ROC) curve analysis based on the one-year primary endpoint and determined as AA-induced platelet aggregation >20%, which is in line with a previous publication.[11] DAPR was defined as exhibiting both HAPR and HCPR.

The VerifyNow®-System

The VerifyNow®-System is a whole blood cartridge-based method to determine the magnitude of platelet agglutination, induced by either AA in the Aspirin assay or ADP and prostaglandin E_1 in the P2Y12 Assay.[30,31] The results are reported in aspirin reaction units (ARU) and P2Y12 reaction units (PRU), respectively. The cut-off used for the VerifyNow P2Y12 assay to identify patients with HCPR was 236 PRU[10], which is in accordance with previous studies.[7,14] The cut-off used for the VerifyNow Aspirin assay to define HAPR was calculated by receiver-operator characteristic (ROC) curve analysis based on the one-year primary endpoint and was determined as 454 ARU. DAPR was defined as exhibiting both HAPR and HCPR.

Statistical analysis

Continuous variables are presented as mean (SD). Categorical data are reported as frequencies (percentages). The chi-square test was used to compare categorical data, including the four groups stratified according to platelet reactivity. Normally distributed continuous variables were compared with a two-sided Student *t*-test.

Logistic regression modelling was used to identify independent correlates of the primary endpoint and to adjust for potential confounders (classic cardiovascular risk factors, renal failure, left ventricular ejection fraction <45%, total stent length, number of lesions treated, amount of stents implanted, bifurcation lesions, co-medication [including use of clopidogrel loading dose, coumadins, proton pump inhibitors, calcium channel blockers, statins or GP IIb/IIIa inhibitors], laboratory parameters [hemoglobin, platelet count and mean platelet volume] and left anterior descendens coronary artery (LAD) or graft-stenting). Platelet reactivity status was entered as a dichotomous variable; patients exhibiting HAPR, HCPR and the interaction term DAPR. All univariate variables with a p-value <0.10 were included in multivariable analysis.

Platelet reactivity status was added to a model including clinical and procedural risk factors. Whether platelet reactivity status according to the various platelet function tests independently contributed to a logistic regression model containing clinical and procedural risk factors, was tested with the likelihood-ratio test. The Hosmer-Lemeshow goodness-of-fit test was performed to assess the adequacy of the model. Survival analysis for patients with and without DAPR according to the specific definitions was performed using the Kaplan-Meier method and differences between the groups were compared using the log-rank test. All statistical analyses were performed with R

(version 2.9, http://www.r-project.org) and a two-tailed p-value of <0.05 was considered significant.

RESULTS

Primary analysis

One-thousand-and-sixty-nine consecutive patients were enrolled, of whom 951 were on aspirin ≥10 days and clopidogrel. Owing to irregularities in platelet assay supply, as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient. For the LTA both AA-induced and ADP-induced aggregation data were available in a total of 921 patients using 5 µmol/L ADP (LTA5) and in 923 patients using 20 µmol/L ADP (LTA20). Furthermore, since the VerifyNow® Aspirin cartridge was started to be used halfway through the POPular-study, the VerifyNow Aspirin-assay was performed in less than half of the population. In a total of 422 patients both the Aspirin-assay and the P2Y12- Assay were performed.

Baseline characteristics of the total cohort are shown in **Table 1**. Clinical outcome at 12 months was available for 949 (99.9%) of the patients. During one-year follow-up 78 (8.2%) patients suffered from the primary endpoint; a total of 16 (1.7 %) patients died, 54 (5.7%) patients had non-fatal acute myocardial infarction, 9 (0.9%) patients presented with definite stent thrombosis and 11 (1.2 %) patients suffered from non-fatal ischemic stroke.

LTA and clinical outcome

The incidence of HCPR was 14.9% using LTA5 and 13.0% using LTA20 (**Table 2A**). The prevalence of HAPR was up to 2 fold higher in patients exhibiting HCPR as compared to patients without HCPR (p<0.0001) (**Table 2B**) and approximately a quarter of the patients exhibited DAPR (**Table 2A**).

Table 3 summarizes one-year clinical outcome for patients without high on-treatment platelet reactivity, with HAPR, HCPR or DAPR according to the different platelet function tests. When measured with LTA5 or LTA20, patients with DAPR had numerically (but not statistically significant) more events than patients with HCPR, or HAPR. Patients with HCPR, HAPR or DAPR had similarly increased rates of the primary endpoint as compared to patients without HPR (**Table 3**). Kaplan-Meier analysis demonstrated that the overall risk for the composite endpoint was significantly higher in patients with isolated HCPR, isolated HAPR or DAPR as compared to patients without high on-treatment platelet reactivity using LTA5 and LTA20 (**Figure 1**).

VerifyNow and clinical outcome

The incidence of DAPR according to the VerifyNow was 14.7% (**Table 2A**) and the prevalence of HAPR was 2.5 fold higher in patients exhibiting HCPR as compared to patients without HCPR (p<0.0001) (**Table 2B**). Patients with DAPR according to the VerifyNow®-system had the highest incidence of the composite primary endpoint, whereas patients with isolated HCPR or HAPR were not at significantly higher risk as compared to patients without high on-treatment platelet reactivity (**Table 3**). DAPR was independently associated with an increased risk of the primary endpoint (**Figure 2**). Kaplan-

Table 1 Baseline characteristics

Clinical parameters	Total population (n=951)	Population in which all tests are performed (n=410)	p-value
Age	64 ± 10.6	64 ± 11.3	0.99
Gender (male)	717/951 (75.4%)	300/410 (73.2%)	0.42
Hypertension	737/951 (77.5%)	297/410 (72.4%)	0.05
Hypercholesterolemia	769/951 (80.9%)	318/410 (77.6%)	0.19
Diabetes Mellitus	175/951 (18.4%)	71/410 (17.3%)	0.70
Family history	580/951 (61.0%)	230/410 (56.1%)	0.09
Current smoking	107/951 (11.3%)	39/410 (9.5%)	0.39
LVEF<45%	133/951 (14.0%)	52/410 (12.7%)	0.55
Renal insufficiency	72/951 (7.6%)	34/410 (8.3%)	0.66
Prior myocardial infarction	519/951 (54.6%)	239/410 (58.3%)	0.21
Medication			
Loading dose clopidogrel	489/951 (51.4%)	193/410 (47.1%)	0.16
PPI	270/951 (28.4%)	127/410 (31.0%)	0.36
Coumarin derivates	24/951 (2.5%)	11/410 (2.7%)	0.85
Laboratory Parameters			
Platelet count (x10^9)	273.4 ± 78.8	268.8 ± 78.4	0.32
WBC (x10^9)	7.7 ± 2.3	7.7 ± 2.4	0.99
Hemoglobin (g/dL)	13.8 ± 3.5	14.0 ± 5.0	0.24
Procedural Parameters			
Mean no.of stents implanted	1.58	1.53	0.40
Minimal stent diameter (mm)	3.1 ± 0.8	3.1 ± 1.1	0.99
Total stent length (mm)	28.3 ± 17.1	27.1 ± 15.6	0.22
Bifurcation lesion	32/951 (3.4%)	9/410 (2.2%)	0.30
Drug eluting stent	604/946 (63.8%)	263/410 (64.1%)	0.95
LAD	450/951 (47.3%)	206/410 (50.2%)	0.34

LVEF = left ventricular ejection fraction; MI = myocardial infarction; PCI= percutaneous coronary intervention; PPI =proton pump inhibitors; CCB = calcium channel blockers; WBC = white bloodcell count, LAD = Left Anterior Descending Artery

Definitions

Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.
Hypercholesterolemia: *A fasting LDL-cholesterol ≥ 3.4 mmol/L or being* on statin therapy at the time of inclusion.
Diabetes mellitus: According to the World Health Organization criteria (37)
Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).
Renal insufficiency: Creatinin > 120 µmol/L

Meier analysis demonstrated that the overall risk for the composite endpoint was significantly higher in patients with DAPR as assessed by the VerifyNow®-System compared to patients without high on-treatment platelet reactivity (**Figure 1**).

Table 2A: Platelet reactivity status according to the various platelet function tests

	NPR	HCPR	HAPR	DAPR
LTA 5 (n=921)	342 (37.1%)	137 (14.9%)	194 (21.1%)	248 (26.9%)
LTA 20 (n=923)	360 (39.0%)	120 (13.0%)	228 (24.7%)	215 (23.3%)
VerifyNow (n=422)	218 (51.7%)	106 (25.1%)	36 (8.5%)	62 (14.7%)

Table 2B: Incidence of high on-aspirin platelet reactivity in patients with versus patients without high on-clopidogrel platelet reactivity

	HAPR in patients without HCPR	HAPR in patients with HCPR	p-value
LTA 5	194/536 (36.2%)	248/385 (64.4%)	<0.0001
LTA 20	228/588 (38.8%)	215/335 (64.2%)	<0.0001
VerifyNow	36/254 (14.3%)	62/168 (36.9%)	<0.0001

LTA = light transmittance aggregometry; ADP = adenosine diphosphate; NPR = neither high on-clopidogrel platelet reactivity nor high on-aspirin platelet reactivity; HAPR = high on-aspirin platelet reactivity; HCPR = high on-clopidogrel platelet reactivity; DAPR = high on-clopidogrel platelet reactivity and high on-aspirin platelet reactivity.

Table 3: Clinical outcome

	LTA 5 NPR n=342	HCPR n=137	HAPR n=194	DAPR n=248	p-value
Death, MI, ST, stroke	14 (4.1%)	15 (10.9%)*	17 (8.8%)†	28 (11.3%)‡	0.006
Death	2 (0.6%)	2 (1.5%)	4 (2.1%)	7 (2.8%)§	0.19
MI	10 (2.9%)	11 (8.0%)‖	11 (5.7%)	20 (8.1%)#	0.03
ST	1 (0.3%)	2 (1.5%)	4 (2.1%)	2 (0.8%)	0.22
Stroke	3 (0.9%)	3 (2.2%)	2 (1.0%)	2 (0.8%)	0.60

	LTA 20 NPR n=360	HCPR n=120	HAPR n=228	DAPR n=215	p-value
Death, MI, ST, stroke	15 (4.2%)	14 (11.7%)**	22 (9.6%)††	23 (10.7%)‡‡	0.006
Death	3 (0.8%)	1 (0.8%)	7 (3.1%)	4 (1.9%)	0.18
MI	10 (2.8%)	11 (9.2%)§§	12 (5.3%)	19 (8.8%)‖‖	0.006
ST	1 (0.3%)	2 (1.7%)	3 (1.3%)	3 (1.4%)	0.38
Stroke	3 (0.8%)	3 (2.5%)##	4 (1.8%)	0 (0.0%)	0.12

	VerifyNow NPR n=218	HCPR n=106	HAPR n=36	DAPR n=62	p-value
Death, MI, ST, stroke	9 (4.1%)	10 (9.4%)	2 (5.6%)	11 (17.7%)***	0.004
Death	3 (1.4%)	2 (1.9%)	0 (0.0%)	4 (6.5%)†††	0.07
MI	5 (2.3%)	8 (7.5%)‡‡‡	2 (5.6%)	4 (6.5%)	0.15
ST	0 (0.0%)	1 (0.9%)	1 (2.8%)	1 (1.6%)	0.21
Stroke	2 (0.9%)	0 (0.0%)	0 (0.0%)	3 (4.8%)$^{§§§, ||||||}$	0.03

MI = myocardial infarction; ST = Stent thrombosis

LTA 5 *p=0.009, †p=0.033, ‡p=0.001, §p=0.040, $^{||}$p=0.023, $^{\#}$p=0.007; all vs. NPR
LTA 20 **p=0.006, †† p=0.009, ‡‡ p=0.003, §§ p=0.007, $^{||||}$ p=0.002; all vs NPR and $^{\#\#}$ p=0.045 vs DAPR
VerifyNow® *** p=0.001, ††† p=0.045, ‡‡‡ p=0.033, all vs. NPR, §§§ p=0.049 vs HCPR and $^{||||||}$ p=0.048 vs HAPR

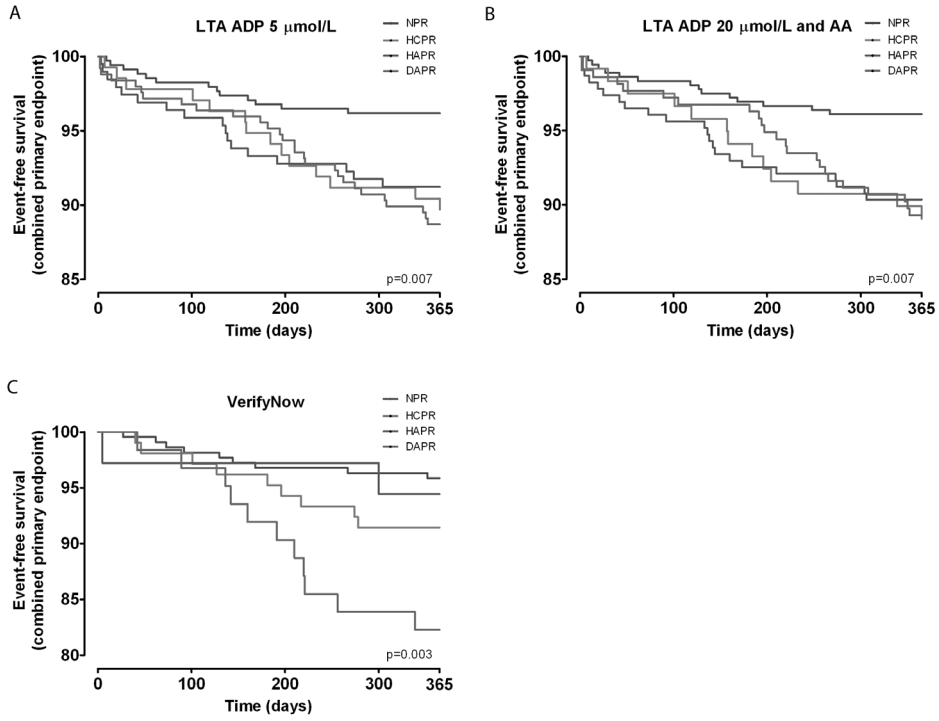

Figure 1: Kaplan-Meier Analysis
Kaplan Meier analysis for the event rate of the combined primary endpoint in patients with and without high on-treatment platelet reactivity as measured by the multiple platelet function assays.
LTA = light transmittance aggregometry; ADP = adenosine diphosphate; NPR = neither high on-clopidogrel platelet reactivity nor high on-aspirin platelet reactivity; HAPR = high on-aspirin platelet reactivity; HCPR = high on-clopidogrel platelet reactivity; DAPR= high on-clopidogrel platelet reactivity and high on-aspirin platelet reactivity.

P-values of log-rank test for differences in event rate of the combined primary endpoint between groups stratified by platelet reactivity status (NPR, HAPR, HCPR and DAPR).

LTA 5	NPR	HCPR	HAPR
HCPR	0.005		
HAPR	0.026	0.53	
DAPR	0.009	0.94	0.42

LTA 20	NPR	HCPR	HAPR
HCPR	0.003		
HAPR	0.008	0.58	
DAPR	0.003	0.75	0.76

VerifyNow®	NPR	HCPR	HAPR
HCPR	0.006		
HAPR	0.69	0.47	
DAPR	0.0002	0.12	0.09

Predictive model

Logistic regression modelling was used to identify independent predictors for the primary endpoint (**Figure 2**). Platelet reactivity status according to LTA5 and LTA20 was an independent predictor of the primary endpoint (HCPR; HAPR as well as DAPR). DAPR as assessed by the VerifyNow®-System was the strongest independent predictor of the primary endpoint. Other variables independently associated with the 1-year primary endpoint were age (calculated for an increase of 10 years), hypertension, a LVEF < 45%, graft-stenting and a bifurcation lesion. The addition of platelet reactivity status to the predictive model consisting of these clinical and procedural risk factors revealed that platelet reactivity as measured with light transmittance aggregometry (both LTA5 and LTA20) and the VerifyNow®-System significantly improved the area under the ROC-curve. Likewise, the likelihood-ratio test demonstrated that platelet reactivity status according to these tests had additional contribution to the model (**Table 4**). The goodness-of-fit test demonstrated that the predicting model was adequate (all p-values>0.20).

Secondary analysis

In 410 patients LTA5, LTA20 and VerifyNow results were available**.** During one-year follow-up 29 (7.1%)patients suffered from the primary endpoint; a total of 8 (2.0 %) patients died, 17 (4.1%) patients had non-fatal acute myocardial infarction, 3 (0.7%) patients presented with definite stent thrombosis and 5 (1.2 %) patients suffered from non-fatal ischemic stroke.

The addition of platelet reactivity status to the predictive model consisting of clinical and procedural risk factors revealed that DAPR as measured with the VerifyNow®-System significantly improved

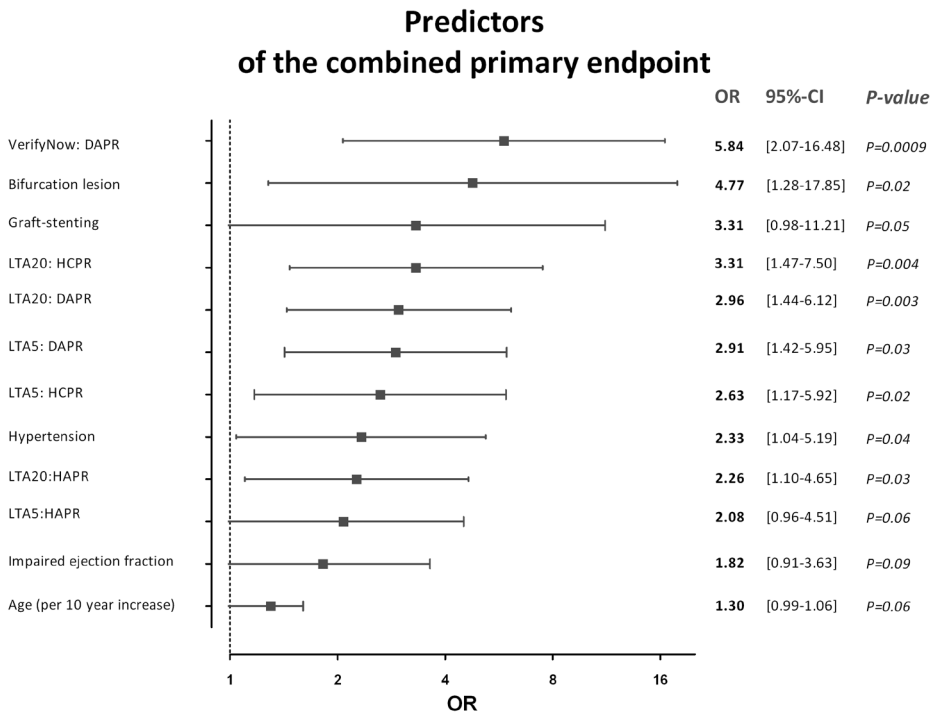

Figure 2: Independent predictors of the primary endpoint
Independent risk factors of the primary endpoint.
Clinical and procedural factors and platelet reactivity status according to the various Platelet function tests.

DAPR= high on-clopidogrel platelet reactivity and high on-aspirin platelet reactivity; HAPR = high on-aspirin platelet reactivity; HCPR = high on-clopidogrel platelet reactivity; HAPR = high on-aspirin platelet reactivity; LTA5= AA and 5 µmol/L ADP-induced light transmittance aggregometry; LTA20= AA and 5 µmol/L ADP

Table 4: Area under the curve

	AUC	p-value for addition*
Model 1: Classical cardiovascular risk factors†	0.66	Reference
Model 2: Model 1 + procedural risk factors‡	0.73	0.0001
Model 3: Model 2 + residual platelet reactivity		
- DAPR as measured with AA- and 5 mmol/L ADP-induced LTA	0.75	0.015
- DAPR as measured with AA- and 20 mmol/L ADP-induced LTA	0.75	0.006
- DAPR as measured with the VerifyNow Aspirin and P2Y12 assays	0.80	0.009

AUC of different backward regression models for the prediction of the primary end point at one-year follow-up
† Age, hypertension, hypercholesterolemia, LVEF (left ventricular ejection fraction) < 45%, prior CABG
‡ Total stent length, no. of lesions treated, no. of stents implanted, LAD- stenting, graft-stenting, bifurcation lesion, clopidogrel loading dose vs maintenance dose.
* Likelihood Ratio Test for additional value of platelet reactivity status (increase in AUC) as measured with multiple platelet function tests.

Table 5: Area under the curve
In the subpopulation of patients in whom all platelet function tests are available

	AUC	p-value for addition*
Model 1: Classical cardiovascular risk factors†	0.68	Reference
Model 2: Model 1 + procedural risk factors‡	0.75	0.20
Model 3: Model 2 + residual platelet reactivity		
- DAPR as measured with AA- and 5 mmol/L ADP-induced LTA	0.77	0.20
- DAPR as measured with AA- and 20 mmol/L ADP-induced LTA	0.77	0.33
- DAPR as measured with the Aspirin and P2Y12 assays of VerifyNow	0.78	0.005

AUC of different backward regression models for the prediction of the primary end point at one-year follow-up
† Age, hypertension, hypercholesterolemia, LVEF (left ventricular ejection fraction) < 45%, prior CABG
‡ Total stent length, no. of lesions treated, no. of stents implanted, LAD- stenting, graft-stenting, bifurcation lesion, clopidogrel loading dose vs maintenance dose.
* Likelihood Ratio Test for additional value of platelet reactivity status (increase in AUC) as measured with multiple platelet function tests.

the area under the ROC-curve in this subpopulation (**Table 5**). The goodness-of-fit test confirmed that the predicting model was adequate (p-value=0.25). In contrast, DAPR as assessed by LTA did not improve the predictive ability of the model and irrespective of the platelet function assay used neither did HCPR or HAPR increase the predictability.

DISCUSSION

The principal finding of the present study is that dual high on-treatment platelet reactivity (DAPR) occurs with varying prevalence between 14.7% and 26.9% according to the platelet function assay used and is more prevalent than previously assumed[15-18]. Of even more importance, DAPR is associated with the occurrence of atherothrombotic events and is a better predictor of adverse outcome than isolated high on-clopidogrel or high on-aspirin platelet reactivity.

Most studies to date evaluated the magnitude of platelet reactivity in response to a single antiplatelet drug (either aspirin or clopidogrel) and therefore the observed higher risk for recurrent events may partly have been due to dual high on-treatment platelet reactivity.[3-14] Measurement of platelet reactivity to multiple agonists comprises the efficacy of dual antiplatelet therapy and may potentially be a more comprehensive method to assess the future risk of an individual patient undergoing PCI.

The mechanisms behind the high interindividual variability in response to both aspirin and clopidogrel are manifold and include baseline individual variability, genetic polymorphisms and clinical factors.[32,33,27,34,35,36] The fact that the vast majority of patients exhibiting high on-clopidogrel platelet reactivity also exhibit high on-aspirin platelet reactivity is in accordance with previous studies[37,38] and might be explained by a mechanistic interdependence of the different pathways involved in platelet reactivity, since the P2Y12-receptor potentiates the generation of thromboxane-A_2[39-41]. Another plausible explanation is that those patients with DAPR exhibit a

generally higher baseline (intrinsic) platelet reactivity status.[42-45] A third explanation might be that in patients with high on-treatment platelet reactivity an increased platelet turnover leads to the release of young platelets that are not inhibited, since both aspirin and clopidogrel have a short half-life.[46,47]

In our study both light transmittance aggregometry and the VerifyNow®-System were used. In line with previous studies,[3,4,6,10] light transmittance aggregometry was able to discriminate between patients with and without atherothrombotic events at one-year follow-up. However, light transmittance aggregometry, although considered the gold standard for platelet function testing, has a poor reproducibility and is labour intensive and thus not suitable for daily clinical use.[48] We therefore also used the fully-automated point-of care VerifyNow®-System to compare its predictability to light transmittance aggregometry. In our study DAPR according to the VerifyNow®-System had the largest increase in predictability for the occurrence of adverse events. In addition, a secondary analysis in those patients in whom all platelet function assays were performed (n=410), demonstrated that LTA lost its predictive ability, whereas the VerifyNow®-System remains predictive. Therefore, the VerifyNow®-System might be considered a better test to predict clinical events in patients undergoing elective PCI.

Multiple studies have been linking a high on-treatment platelet reactivity to atherothrombotic events and many thresholds to identify patients at higher risk have been established. It has been hypothesized that individual monitoring of platelet reactivity and decreasing the magnitude of platelet reactivity below this threshold might improve clinical outcome.[49,50] Three small studies indeed suggested that individualizing therapy based on platelet function might improve outcome.[9,51,52,20] The only randomized study thus far is the GRAVITAS (Gauging Responsiveness With a VerifyNow® Assay-IMPACT on Thrombosis and Safety-study) a prospective, randomized, three-arm, multi-center trial enrolling 5429 patients undergoing PCI with DES implantation. Patients exhibiting high on-treatment platelet reactivity 12-24 hours post-PCI (n=2214) were randomized to either standard maintenance therapy (75 mg) or to an additional loading dose of 600 mg and a double maintenance dose (150mg). The GRAVITAS demonstrated no benefit of doubling the clopidogrel dose in preventing cardiovascular events in patients with high on-clopidogrel platelet reactivity. Still, this study does not rule out the benefit of tailoring therapy based on platelet-function testing. On the contrary, since doubling the dose resulted in only a modest reduction of platelet reactivity, these results suggest that the strategy of a double dose of clopidogrel is ineffective in this low-risk population.[53] Tailoring therapy based on the use of novel, more potent antiplatelet medication (ie prasugrel or ticagrelor) might be more effective. The latter is the subject of investigation in the currently ongoing TRIGGER-PCI (NCT00910299) randomizing to prasugrel versus clopidogrel. The logical drawback of efficient platelet inhibition is the risk of bleeding complications and it has been suggested that measuring platelet function might be the solution to define a therapeutic window between bleeding and thrombotic events. Taking the costs and risks associated with bleeding into account, we consider platelet function testing a better option as compared to prescribing all

patients more potent antiplatelet therapy. In that case, point-of-care platelet function testing is obviously preferred.[54]

Some limitations merit mention. First, the present analysis of DAPR has a decreased statistical power due to a smaller sample size than used in POPular and the stratification into four categories (patients without high on-treatment platelet reactivity; with HAPR, HCPR or DAPR) instead of two (patients without high on-treatment platelet reactivity vs. those exhibiting high on-treatment platelet reactivity). This is most apparent when platelet reactivity status was assessed by the VerifyNow®-System, since the VerifyNow® Aspirin assay was performed in only half of the patients who were tested with the VerifyNow® P2Y12 cartridge. The absence of a higher risk in patients with isolated high on-aspirin platelet reactivity or isolated high on-clopidogrel platelet reactivity as measured with LTA might be attributed to this smaller number of patients. This is further elucidated in the secondary analysis in which LTA lost its predictive ability as well. Second, not all of the currently available platelet function tests were included. Third, single time point assessment represent a common limitation to most studies assessing the prognostic value of platelet function testing, including the present one. Last, patients received three different, adequate, clopidogrel doses. Although previous studies have demonstrated differences in the effect on platelet reactivity of these three dosing regimens, these three regimens are daily clinical practice, and the present analysis thus reflects the clinical relevance of monitoring platelet function in daily care.

In conclusion, one in 5 patients undergoing elective PCI exhibits high on-treatment platelet reactivity to both aspirin and clopidogrel. These patients are at higher risk for atherothrombotic events than those with high on-treatment platelet reactivity to either aspirin or clopidogrel. Dual high on-treatment platelet reactivity when measured with the point-of care VerifyNow®-System might have a higher predictability for atherothrombotic events as compared to measured by light transmittance aggregometry.

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Chapter 6

Influence of high-on treatment platelet reactivity on clinical outcome in patients with diabetes mellitus undergoing percutaneous coronary intervention. Reason to intensify platelet inhibition?

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INTRODUCTION

Patients with diabetes mellitus (DM) are at high risk for atherothrombotic events and exhibit a higher magnitude of platelet reactivity as compared to non-diabetics.

Although the efficacy of dual antiplatelet therapy has been established in the prevention and treatment of atherothrombotic events,[1,2] aspirin and clopidogrel appear to have less effect in diabetic patients.[3-5] Since there are little data on the influence of a lower degree of platelet inhibition and high on-treatment platelet reactivity (HPR) on clinical outcome in diabetic patients, the aim of the present study was to assess platelet function profiles in diabetic patients and to evaluate the impact of HPR on clinical outcome in this cohort.

METHODS

A prospective cohort study of 1069 consecutive patients undergoing elective percutaneous coronary intervention (PCI) with stent-implantation was performed.[6] Of these, 179 were diabetic patients either on insulin or oral hypoglycaemic medication. All patients received clopidogrel treatment (600 mg loading dose > 4 hours, 300 mg loading dose >24 hours or 75 mg at least 5 days) before PCI and all patients were on aspirin at a dose of 80 to 100 mg daily ≥10days, unless they were on long-term anticoagulation with coumarin derivates. The clinical endpoint was defined as a composite of all-cause death, non-fatal myocardial infarction, definite stent thrombosis and ischemic stroke. On-treatment platelet reactivity was determined using light transmittance aggregometry (LTA) induced by adenosine diphosphate in final concentrations of 5 and 20μmol/L and the VerifyNow P2Y12-assay. HPR was defined according to the cut-offs established in the POPular study.[6]

Statistical analysis

Survival curves for patients with and without HPR were generated by the Kaplan Meier method and the difference among groups was compared using the log-rank test. Odds ratios (OR) and 95% confidence intervals (CI) for the occurrence of clinical endpoints were also calculated. A two-sided p value < 0.05 was considered significant (SPSS-version 17.0).

RESULTS

Patients with DM had a significantly higher on-clopidogrel platelet reactivity as compared to patients without DM (42.9±14.1% vs. 39.2±14.7%, p=0.001 using 5 μmol/L ADP-induced LTA, 61.6±13.9% vs. 56.8±14.7%, p<0.001 using 20 μmol/L ADP-induced LTA, 233±74 vs. 204±75 , PRU, p<0.001 using the VerifyNow P2Y12-assay). In addition, the incidence of HPR in patients with DM was significantly higher than in patients without DM and varied depending on the platelet function test used (106/194[54.6%] vs. 340/855[39.8%], p=0.0002 using 5 μmol/L ADP; 95/195[48.7%] vs. 301/856[35.2%], p=0.0004 using 20 μmol/L ADP and 104/194[53.6%] vs. 306/858[35.7%], p<0.0001 using the VerifyNow P2Y12®-assay).

The event rate was lower in diabetics as compared to patients without diabetes mellitus (14/165[7.8%]

vs.119/751 [13.7%], p=0.04). Furthermore, in the diabetic subpopulation, the frequency of the composite endpoint, as well as its single components, was similar between patients with and without HPR, regardless of the test used. **(Table 1)** In addition, Kaplan Meier analysis demonstrated that the cumulative survival free from the primary endpoint was similar between diabetic patients with and without HPR (all p-values >0.10) **(Figure 1)**.

Table 1: Clinical outcome

Platelet function test				
LTA (5 µM ADP)	**NPR (n=94)**	**HPR (n=84)**	**OR (95% CI)**	**p-value**
Combined endpoint	7/94 (7.5%)	7/84 (8.3%)	1.15 (0.38-3.41)	0.81
All-cause mortality	0/94 (0.0%)	1/84 (1.2%)	0	0.47
Nonfatal Myocardial infarction	4/94 (4.2%)	5/84 (6.0%)	1.44 (0.37-5.56)	0.59
Stent thrombosis	1/94 (1.1%)	0/84 (0.0%)	0.53 (0.46-0.61)	0.35
Ischemic stroke	3/94 (3.2%)	1/84 (1.2%)	0.37 (0.04-3.63)	0.38
LTA (20 µM ADP)	**NPR (n=105)**	**HPR (n=73)**	**OR (95% CI)**	**p-value**
Combined endpoints	7/105 (6.7%)	7/73 (9.6%)	1.49 (0.50-4.43)	0.45
All-cause mortality	0/105 (0%)	1/73 (1.4%)	0	0.23
Nonfatal Myocardial infarction	5/105 (4.8%)	4/73 (5.5%)	1.16 (0.31-4.47)	0.83
Stent thrombosis	1/105 (1.0%)	0/73 (0.0	0.59 (0.52-0.67)	0.40
Ischemic stroke	2/105 (1.9%)	2/73 (2.7%)	1.46 (0.20-10.54)	0.71
VerifyNow P2Y12	**NPR (n=91)**	**HPR (n=83)**	**OR (95% CI)**	**p-value**
Combined endpoints	5/91 (5.5%)	10/83 (12.0%)	2.36 (0.77-7.21)	0.12
All-cause mortality	1/91 (1.1%)	1/83 (1.2%)	1.10 (0.07-17.83)	0.95
Nonfatal Myocardial infarction	3/91 (3.3%)	6/83 (7.2%)	2.29 (0.55-9.45)	0.24
Stent thrombosis	1/91 (1.1%)	0/83 (0.0%)	0.52 (0.45-0.60)	0.34
Ischemic stroke	1/91 (1.1%)	3/83 (3.6%)	3.38 (0.34-33.10)	0.27

HPR = high on-treatment platelet reactivity according to the defined cut-off (i.e. ≥42,9% using 5 µmol/L ADP-induced LTA; ≥64.5% using 20 µmol/L ADP-induced and ≥236 PRU using the VerifyNow P2Y12®-assay); NPR = normal on-treatment platelet reactivity

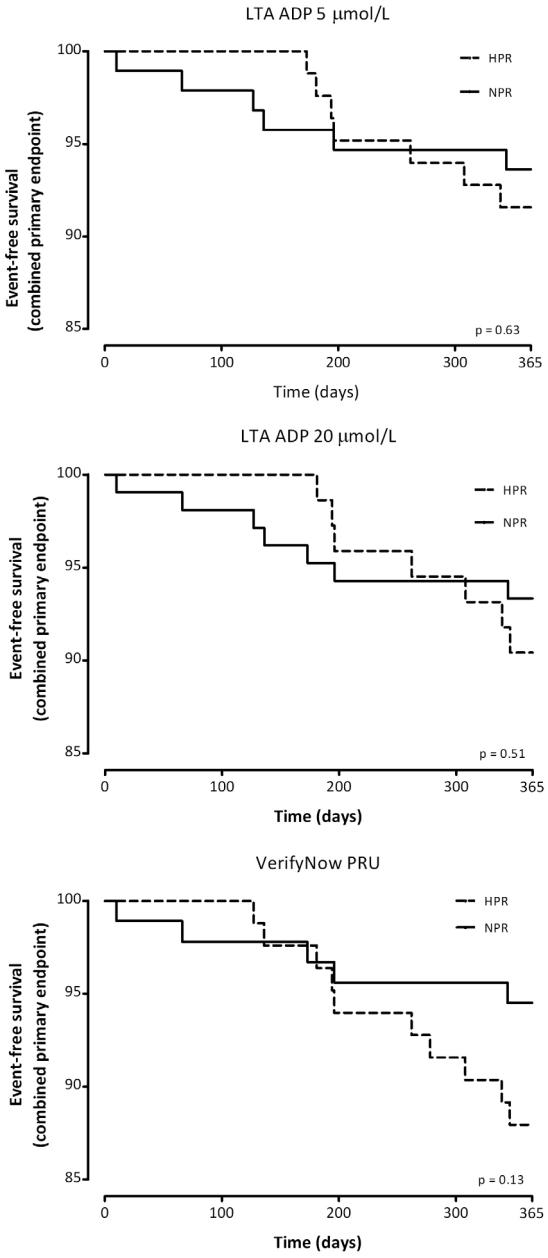

Figure 1: Kaplan-Meier Analysis
Kaplan-Meier analysis for the event rate of the combined primary endpoint in patients with and without high on-treatment platelet reactivity as measured by 5 and 20 µmol/L ADP-induced LTA and the VerifyNow P2Y12®-assay.
HPR = high on-treatment platelet reactivity according to the defined cut-off (i.e. ≥42,9% using 5 µmol/L ADP-induced LTA; ≥64.5% using 20 µmol/L ADP-induced and ≥236 PRU using the VerifyNow P2Y12®-assay). NPR = normal on-treatment platelet reactivity

DISCUSSION

Nowadays most coronary lesions are amenable to PCI. It is however questionable whether patients with DM are optimal candidates for PCI because the event rate in patients with DM is deemed to be higher.[7] This increased event rate may be due to higher platelet reactivity despite optimal antiplatelet medication. Estimating patients mortality risk following either surgery or PCI remains important to make an informed clinical decision on the best possible revascularization approach. Numerous efforts have been made to estimate the risk of morbidity and mortality following PCI and multiple risk-adjustment models have been developed. The EuroSCORE, which includes 17 patient-, cardiac- and surgical procedure-related variables and has been originally validated to predict perioperative surgical mortality, was recently shown to be an independent predictor of major adverse cardiac events (MACEs) in studies with both percutaneous and surgical treatment arms.[8,9] In addition, the SYNTAX score, based on angiographic lesion complexity, has been shown to be an independent predictor of MACE in patients treated with PCI, but not with coronary artery bypass grafting.[10] Recently, the National Cardiovascular Database Registry (NCDR CathPCI) risk score, with multiple pre-procedural risk factors, has been prospectively validated in PCI-patients.[11]

Remarkably, however, none of these scores comprised the presence of diabetes mellitus as risk factor. So, although patients with DM have a two- to fourfold increased risk of cardiovascular disease as compared to non-diabetic individuals and up to 75% of these patients will eventually die of atherothrombotic events,[12,13], in our study they do not appear to have a higher risk during and directly following revascularisation. This suggests that diabetes mellitus is not a risk factor predicting events after revascularisation in the selected patient group undergoing PCI. A multidisciplinary decision-making process in the heart team seems capable to determine the safety and likelihood of effective revascularization with either PCI or CABG or to prefer optimal medical therapy, thereby reducing the risk following intervention in diabetics.[14]

The same observation applies to the present analysis. High on-treatment platelet reactivity is more prevalent in patients with diabetes mellitus than in a population including non-diabetics. However, in diabetic patients with established coronary artery disease undergoing elective PCI, assessment of platelet reactivity was not associated with significant rates of death, myocardial infarction, stent thrombosis or stroke, regardless of the test used. This finding is in line with a meta-analysis, in which high on-treatment platelet reactivity was associated with a significantly higher event rate in the cohort without diabetes but not in that with diabetes.[15] In addition, the event rate is lower in diabetics as compared to patients without diabetes mellitus.

Given the high platelet reactivity levels in diabetic patients, it has been suggested that intensified antiplatelet therapy might be beneficial. However, the majority of studies were unable to demonstrate an advantage of more potent or additional antiplatelet therapy. Although the diabetic subanalysis of the TRITON-TIMI 38-trial demonstrated that the benefit of prasugrel over clopidogrel in reducing the primary endpoint (consisting of cardiac mortality, myocardial infarction or stroke) was greater in diabetic patients, randomization was not stratified by diabetic status and there was

no significant interaction between patients with and without diabetes.[16] In addition, the PLATO-trial (comparing ticagrelor to clopidogrel) showed that ticagrelor reduced the primary endpoint in diabetic patients by 2.1% (23% relative) without reaching statistical significance.[17] Similarly, the CURE-trial (comparing clopidogrel to placebo in unstable angina) and the CURRENT OASIS 7 trial (comparing high- dose to low-dose clopidogrel in patients undergoing PCI) no significant interactions for diabetic status were found with respect to the primary endpoints. In addition,[18,19] the ISAR SWEET study investigating the addition of abciximab on top of dual antiplatelet therapy with aspirin and clopidogrel, demonstrated no clinical benefit of this intensified antiplatelet therapy in a subpopulation of diabetic patients undergoing PCI.[20] So, higher levels of platelet inhibition might not be effective to prevent atherothrombotic events in diabetics. Given the pro-thrombotic state that characterizes diabetes mellitus, some studies suggest other options than platelet inhibition to overcome the supposed higher risk such as the use of anti-thrombin agents.[21] Although the association between high on-treatment platelet reactivity and adverse clinical outcome is well-established,[6,22-24] in the present study its use did not improve classification of individuals into clinically relevant risk categories. This suggests that in patients suffering from diabetes mellitus undergoing *elective* PCI, assessment of platelet reactivity does not improve prediction of clinical outcome.[25]

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Chapter 7

The impact of renal function on platelet reactivity and clinical outcome in patients undergoing percutaneous coronary intervention with stenting

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ABSTRACT

Background: Patients with chronic kidney disease (CKD) have an increased risk of cardiovascular disease. Previous studies have suggested that patients with renal failure have less therapeutic benefit of antiplatelet therapy. There are, however, few data on the relation between renal function and platelet reactivity. The aim of the present study was to determine the influence of an impaired renal function on the magnitude of platelet reactivity and clinical outcome.

Methods and results: On-clopidogrel platelet reactivity was determined in 988 patients on dual antiplatelet therapy, undergoing elective coronary stent implantation, using adenosine diphosphate-induced light transmittance aggregometry (LTA) and the VerifyNow®P2Y12-assay. Patients were divided into two groups according to the presence or absence of moderate/severe CKD (glomerular filtration rate<60ml/min). Furthermore, the incidence of the composite of all-cause death, non-fatal acute myocardial infarction, stent thrombosis and ischemic stroke at one-year follow-up was evaluated.

Patients with CKD (n=180) had significantly higher platelet reactivity, regardless of the platelet function test used. Patients with CKD more frequently had high on-clopidogrel platelet reactivity. The event-rate was the highest in patients with both HCPR and CKD (8.4%[16/87] vs. 4.8%[24/504] in those with neither HCPR nor CKD using the VerifyNow®P2Y12-assay.

Conclusion: Both the magnitude of platelet reactivity as well as the incidence of HCPR was higher in patients with CKD. CKD-patients with HCPR were at the highest risk of long-term cardiovascular events, suggesting the need for intensified antiplatelet therapy in these high-risk patients.

INTRODUCTION

Chronic kidney disease (CKD) is associated with an increased risk of adverse outcomes in patients presenting with an acute coronary syndrome (ACS) or those undergoing percutaneous coronary intervention (PCI), even if renal function is only moderately disturbed.[1,2] Accelerated atherosclerosis, oxidative stress, inflammation and a prothrombotic state have been proposed as possible mechanisms.[3] Dual antiplatelet therapy with aspirin and a thienopyridine has become the standard care in the treatment of patients undergoing coronary stenting and those presenting with ACS.[4,5] Recent findings from placebo-controlled trials suggest that patients with renal function might not accrue equal therapeutic benefit of clopidogrel as compared to patients with a normal renal function.[6,7] However, data on the relationship between renal function and platelet reactivity in patients on dual antiplatelet therapy are scarce.[8,9] Therefore, the aim of the present study is to determine the influence of an impaired renal function on the magnitude of platelet reactivity and clinical outcome in a large cohort of patients on dual antiplatelet therapy undergoing elective coronary stenting.

METHODS

Population and study design

The **POP**ular-study (The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI-study) was a prospective, observational study that enrolled consecutive patients with documented coronary artery disease undergoing elective coronary stenting. The entry- and exclusion-criteria were described in the original publication.[10] In the present sub-analysis all patients were on dual antiplatelet therapy with adequate clopidogrel treatment and low-dose aspirin of 80-100 mg daily for at least 10 days, unless they were on long-term coumarin derivates.

Renal function was defined according to the National Kidney Foundation Classification[11] into normal renal function (creatinine clearance≥ 90 mL/min/1.73 m^2); and into kidney damage with mildly decreased glomerular filtration rate (GFR)(creatinine clearance 60-89 ml/min), moderately decreased GFR (creatinine clearance 30-59 mL/min/1.73 m^2) and severely decreased GFR (creatinine clearance <30 mL/min/1.73 m^2). Creatinine clearance was calculated using the "Modification of diet in renal disease"-formula Modified-4" (MDRD-4). Due to the limited number of patients with severely decreased GFR, these patients were combined with patients having moderate renal failure. To determine the relation between renal function and clinical outcome, patients were categorized into two groups according to the presence or absence of moderate/severe CKD (creatinine clearance <60 mL/min/1.73 m^2). In line with a previous study on this subject, to determine the relation between renal function and platelet function, analyses were performed across three groups (normal renal function, mild CKD, moderate/severe CKD). The study was conducted according to the principles of the Declaration of Helsinki and the laws and regulations applicable in the Netherlands. All patients gave written informed consent.

Blood sampling and platelet function testing

Prior to heparinization, whole blood was drawn from the femoral or radial artery sheath into into Vacuette® tubes (Greiner Bio-one, Frickenhausen, Germany) containing 3.2% sodium citrate for all platelet function tests. Blood samples for whole blood count were drawn into tubes containing K_3-EDTA. Platelet function testing was performed within 2 hours after blood withdrawal.

Laboratory endpoint (platelet function testing)

Light Transmittance Aggregometry (LTA)

Light transmittance aggregometry (LTA) was performed using an APACT 4004 aggregometer (LABiTec, Arensburg, Germany) at 37°C as previously described.[12,13-15] Platelet poor plasma (PPP) was used as a reference for 100% aggregation and maximal platelet aggregation (%) was measured in non-adjusted platelet rich plasma after stimulation with adenosine diphosphate (ADP) in a final concentration of 5 µmol/L to determine on-clopidogrel platelet reactivity. The cut-off to determine high on-clopidogrel platelet reactivity (HCPR) was 42.9% aggregation.[10]

VerifyNow®System

The VerifyNow® (Accumetrics, San Diego, USA) is a whole blood assay designed to measure agonist-induced platelet aggregation. The clopidogrel response was measured using the P2Y12 assay that contains 20 µmol/L ADP to induce P2Y12-dependent platelet aggregation, and 22nmol/L prostaglandin E_1 (PGE_1) to minimize the contribution of the ADP-activated P2Y1-receptor to platelet aggregation.[16,17] Results are described as P2Y12 Reaction Units (PRU) respectively. HCPR was defined as ≥ 236 PRU.[10]

Clinical endpoint

The clinical endpoint was a composite of all-cause mortality, non-fatal myocardial infarction (defined as the occurrence of ischemic symptoms as well as a spontaneous troponin T value or creatine kinase MB greater than the upper limit of normal), definite stent thrombosis (according to the Academic Research Consortium criteria[18]) and ischemic stroke at one-year follow-up. An independent committee, blinded for platelet function data, adjudicated all endpoints through review of source documents of medical records.

Statistical analysis

Continuous variables were expressed as mean ± SD, unless otherwise specified, and categorical variables as frequencies (%). All distributions were checked for normality. Differences in continuous variables were compared by independent *t*-test or Mann-Whitney *U* test, as appropriate. Dichotomous variables were compared by chi-square test or Fisher exact test. One-way analysis of variance was used for comparisons across tertiles according to the severity of CKD and the Tukey-HSD-test was used for comparisons between tertiles.

This study aims to explain-as opposed to predict-the relation between platelet reactivity and chronic

kidney disease. When the objective of a clinical study is to explain, the influence of extraneous determinants should be excluded. Obviously, the randomized controlled clinical trial is the gold standard for this case. In a non-experimental study, confounders can be addressed in a multivariate analysis. [19] Entering a variable that is not a confounder by the strict epidemiological definition, into the multivariate analysis, can affect bias and precision in a harmful way.[20] We consider the knowledge of the nature of the association between CKD and platelet function insufficient to allow a multivariable analysis in order to remedy the influence of confounders. In addition, there is no statistical test for confounding.[21]

Taking into account that this study is not an a priori hypothesis testing study, we decided to present this study in a univariable approach. All data were analyzed with SPSS version 17.0 (SPSS, Chicago, IL) and R (version 2.9http://r-project.org) and a two-sided p-value <0.05 was considered significant.

RESULTS

Patient characteristics

In total 1069 consecutive patients undergoing elective PCI with stent implantation were enrolled. Since data on renal function were missing in 81 of the patients (7.6%), the present study-population consisted of 988 patients. Baseline characteristics of patients with moderate/severe CKD (n=180) and those with a normal renal function or mild CKD (n=808) are depicted in **Table 1**. Patients with moderate/severe CKD were older, more often female and more often suffered from diabetes mellitus. Hypercholesterolemia was less prevalent in patients with moderate/severe CKD and the subsequent use of statins was lower. Furthermore, patients with moderate/severe CKD had a lower haemoglobin, had less often a familial history of coronary artery disease and were less likely to use aspirin.

Relation between renal function and platelet reactivity

Patients with moderate/severe CKD had significantly higher ADP-induced platelet reactivity, regardless of the test used (43.0 ± 14.8% vs. 39.2 ± 14.4%, p=0.002 using 5 µmol/L ADP-induced LTA and 226 ± 82.2 PRU vs. 207 ± 73.8 PRU, p=0.004 using the VerifyNow®System). Patients with moderate/severe CKD were more likely to exhibit HCPR than those without (OR = 2.00; 95%-CI: 1.43-2.83, p=0.0001 using LTA and OR = 1.64; 95%-CI: 1.16-2.30, p=0.005 using the VerifyNow®System).

Platelet function according to severity of CKD

In addition, similar to a previous study[8], analysis of variance (ANOVA) was performed to compare the magnitude of platelet reactivity among three stages of CKD; moderate/severe CKD, mild CKD and normal renal function.

Using 5 µmol/L ADP-induced LTA, ANOVA demonstrated significant higher values of platelet reactivity in patients with moderate/severe CKD as compared to those with a normal renal function or mild CKD. In contrast, no differences in platelet reactivity were observed between patients with a

Table 1: Baseline characteristic

	CKD (n=180)	No CKD (n=808)	p- value
	GFR<60	GRF≥60	
Clinical/demographic data			
Male	101/180 (56.1%)	638/808 (79.0%)	<0.0001
Age (yrs)	71.75 ± 9.15	62.43 ± 10.31	<0.0001
BMI (kg/m^3)	27.35 ± 4.67	27.25 ± 3.85	0.77
Hypertension	146/180 (81.1%)	622/808 (77.0%)	0.23
Hypercholesterolemia	130/180 (72.2%)	656/808 (81.2%)	0.007
Diabetes	44/180 (24.4%)	141/808 (17.5%)	0.03
Current Smoker	13/180 (7.2%)	94/808 (11.6%)	0.09
Family history of CAD	97/180 (53.9%)	501/808 (62.0%)	0.04
Previous MI	91/180 (50.6%)	443/808 (54.8%)	0.30
Previous PCI	57/180 (31.7%)	263/808 (32.6%)	0.82
Previous CABG	25/180 (13.9%)	78/808 (9.7%)	0.09
Co-medication			
Loading dose clopidogrel	88/180 (48.9%)	414/807 (51.3%)	0.56
Aspirin	152/180 (84.4%)	730/807 (90.5%)	0.02
Statin	125/180 (69.44%)	660/807 (81.78%)	<0.0001
Proton pump inhibitor	50/180 (27.8%)	226/807 (28.0%)	0.95
ACE-inhibitor	72/180 (40.0%)	292/807 (36.2%)	0.34
Beta-blocker	127/180 (7056%)	623/807 (77.2%)	0.06
Laboratory data			
Hemoglobine (g/dL)	12.7 ± 1.7	13.8 ± 1.5	<0.0001
Platelet count (· 10^9/L)	278 ± 95.2	272 ± 80.7	0.46
Mean platelet volume (fl)	7.4 ± 0.99	7.5 ± 0.93	0.12

GFR= glomerular filtration rate (using MDRD-4 method); CKD=chronic kidney disease; BMI = Body Mass Index; CAD = Coronary artery disease; MI = Myocardial infarction; PCI = Percutaneous coronary intervention; CABG = Coronary artery bypass graft; PPI = proton pump inhibitor; ACE = Angiotensin-Converting Enzyme.

mildly decreased GFR as compared to those with a normal renal function. (**Figure 1**)

Using the VerifyNow®System, ANOVA established significant higher values of platelet reactivity in patients moderate/severe CKD as compared to those with mild CKD. A trend was shown towards a higher on-treatment platelet reactivity in patients moderate/severe CKD as compared to patients with a normal renal function (p=0.06). In contrast, no differences in platelet reactivity were observed between patients with a mildly decreased GFR as compared to those with a normal renal function.

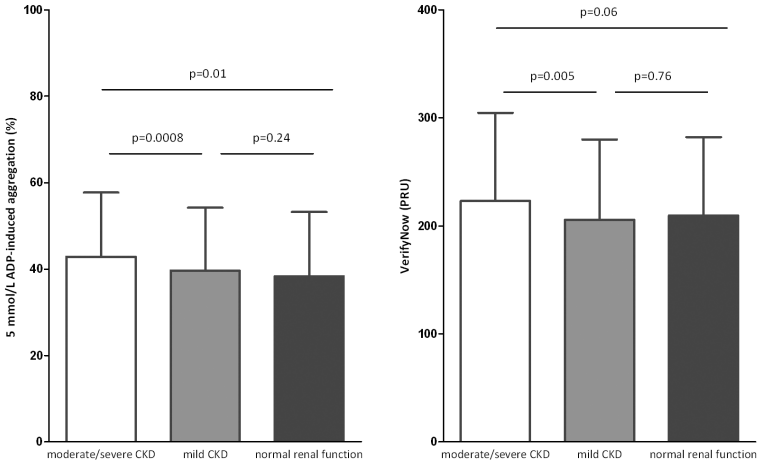

Figure 1: Magnitude of platelet reactivity according to renal function

On-clopidogrel platelet reactivity (mean ± SD) in patients with moderate/severe CKD (GFR<60 ml/min), compared to those without moderate/severe CKD (combining mild CKD [GFR 60-89] and normal renal function [GFR>90]) as assessed by 5 µmol/L ADP-induced LTA (left) and the VerifyNow (right).

Clinical outcome

One-year clinical outcome was available for 986 (99.8%) of the patients. A total of 17 died (1.7%), 60 (6.1 %) patients had non-fatal acute myocardial infarction, 13 (1.3 %) presented with definite stent thrombosis and 13 patients suffered from non-fatal ischemic stroke (1.3 %). **Figure 2** displays the composite endpoint plotted against the presence of HCPR for patients with and without moderate/severe CKD. The cumulative event-rate was the highest in patients with both HCPR and moderate/severe CKD. In addition, the combined endpoint occurred significantly more often in patients with HCPR, both in patients with and without moderate/severe CKD.

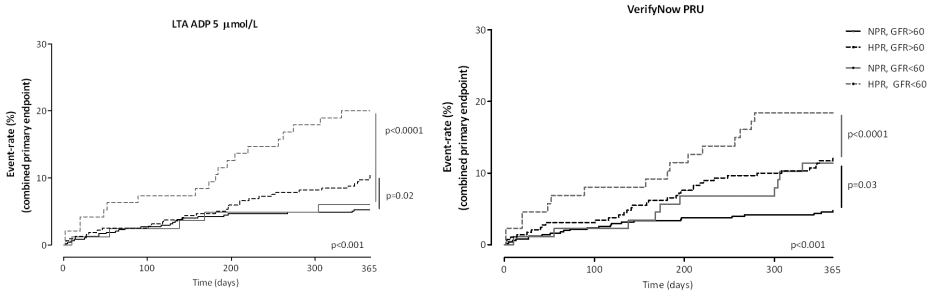

Figure 2: Kaplan Meier Analysis

Kaplan Meier analysis of (A) 5 µmol/L ADP-induced LTA and (B) VerifyNow, demonstrating the event rate in patients with normal on-treatment platelet reactivity (NPR-solid lines) and high on-treatment platelet reactivity (HPR-dotted lines) curve stratified by renal function. Patients with moderate/severe CKD (red lines) and without moderate/severe CKD (combining normal renal function and mild CKD [black lines]) were determined by GFR at baseline.

DISCUSSION

Patients with renal dysfunction, even mild and moderate, have an increased risk of cardiovascular disease. [1,2,22] Although multiple contributing factors, such as accelerated atherosclerosis, oxidative stress, inflammation and a prothrombotic state have been proposed[3,23-25], the physiological mechanism behind the higher event rate remains unclear.

Since platelet reactivity plays a pivotal role in thrombus formation and atherosclerosis,[26,27]dual antiplatelet therapy with both aspirin and clopidogrel has become the mainstay in the treatment of patients undergoing coronary stent implantation and those presenting with acute coronary syndrome (ACS). [4,28] Although previous studies have suggested that patients with renal failure might have less clinical benefit of antithrombotic therapy, few studies have investigated the relation between renal failure and platelet reactivity in the era of dual antiplatelet therapy.[8,9]

The present study provides data on several key questions about the interplay among renal function and clopidogrel pharmacotherapeutics. First, we observed a higher magnitude of platelet reactivity in patients with moderately/severely decreased GFR , thereby confirming the findings from a previous study describing the impact of chronic kidney disease on platelet function profiles in patients with diabetes mellitus.[8] In addition, differences in platelet reactivity appeared only in patients with a moderately/severely decreased GFR, whereas no differences were observed in patients with a normal renal function or a mildly decreased GFR. This supports the observation of a previous study, suggesting a threshold of renal function below which higher platelet reactivity appears, and adds to the findings of clinical studies describing an increase in adverse events in patients with more severe CKD.[23]

Second**,** patients with kidney damage with moderately/severely decreased GFR had a two-fold increase in the likelihood of exhibiting high on-clopidogrel platelet therapy in univariate analysis. The higher incidence of atherothrombotic complications in patients with CKD undergoing hemodialysis was already recognized over 30 years ago[29] and has recently been confirmed in patients with less severe kidney dysfunction.[1] Given the well-established relationship between the magnitude of platelet reactivity and the occurrence of atherothrombotic events,[10,30] efforts to identify those patients with a heightened platelet reactivity status despite adequate dosing of dual antiplatelet therapy have been made.

Patients with both moderate/severe CKD and high on-clopidogrel platelet reactivity were at the highest risk of atherothrombotic events and the combined endpoint occurred significantly more often in patients with HCPR, both in patients with and without moderate/severe CKD. Thus, the present study demonstrates that patients with moderate/severe CKD and HCPR represent an even higher-risk cohort in the CKD-population. In addition the present analysis indicates that in patients suffering from CKD assessment of platelet reactivity does improve prediction of clinical outcome.[31]

Whether patients with an impaired renal function should be treated with novel, more potent antiplatelet agents, remains to be established. Preliminary data on the GRAVITAS (Gauging responsiveness with a VerifyNow assay-impact on thrombosis and safety)-study, enrolling patients

who had undergone an uncomplicated PCI, demonstrated no clinical benefit of doubling the clopidogrel maintenance dose in patients with high on-clopidogrel platelet reactivity.[32] Although the effect of the double dosing on platelet inhibition was only modest and switching to the recently introduced prasugrel or ticagrelor has been advocated, one has to bear in mind that patients with CKD are at increased risk of bleeding as well. [33] Recently, the PLATO (Platelet Inhibition and patient outcomes-trial) demonstrated that in the subgroup of patients with CKD presenting with ACS, ticagrelor is more effective than clopidogrel, without an increase in bleeding. Thus, switching to ticagrelor in patients with moderate/severe CKD and HCPR might be beneficial.[34]

Some limitations merit mention. First, creatinine levels were missing in 81 (7.6%) of the patients. However, the population without samples did not differ from the population with creatinine levels available on most baseline characteristics and outcome measures (data not shown). Second, the number of patients with severe renal failure is small. Thus, it remains unknown whether the findings from the present study can be extrapolated to patients with severe renal failure. Third, single time point assessment represent a common limitation to most studies assessing the prognostic value of laboratory parameters,[35] including the present one. Since platelet function was measured while patients were on dual antiplatelet therapy, it is impossible to establish whether the difference in platelet reactivity should be attributed to a higher instrinsic (baseline) platelet reactivity in patients with renal failure or to a poor responsiveness.

In conclusion, both the magnitude of platelet reactivity as well as the incidence of HCPR was higher in patients with CKD. Patients with both CKD and HCPR were at the highest risk of long-term cardiovascular events, suggesting the need for intensified antiplatelet therapy in these high-risk patients.

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Chapter 8

Effect of gender difference on platelet reactivity

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Submitted

ABSTRACT

Objectives: To compare the magnitude of on-treatment platelet reactivity between genders in patients on dual antiplatelet therapy undergoing elective coronary stenting.

Background: Previous studies have suggested that women do not accrue equal therapeutic benefit of antiplatelet medication as compared to men. The physiological mechanism and clinical implications behind this gender disparity have yet to be established.

Methods: On-treatment platelet reactivity was determined in 717 men and 234 women on dual antiplatelet therapy, undergoing elective coronary stent implantation. Platelet function testing was performed using arachidonic acid and adenosine diphosphate-induced light transmittance aggregometry (LTA) and the VerifyNow P2Y12- and Aspirin-assays. Also the incidence of all-cause death, non-fatal acute myocardial infarction, stent thrombosis and ischemic stroke was evaluated.

Results: Women had higher baseline platelet counts than men. Women exhibited a higher magnitude of on-aspirin platelet reactivity using LTA, but not using the VerifyNow Aspirin-assay. The magnitude of on-clopidogrel platelet reactivity was significantly higher in women as compared to men with both tests used. The cut-off value to identify patients at risk as well as the incidence of clinical endpoints was similar between women and men (16/234[6.8%] vs. 62/717[8.6%], p=0.38).

Conclusion: Although the magnitude of platelet reactivity was higher in women, the absolute difference between genders was small and both the cut-off value to identify patients at risk and the incidence of the composite endpoint was similar between genders. Thus, it is unlikely that the difference in platelet reactivity accounts for a worse prognosis in women.

INTRODUCTION

Coronary artery disease is the main cause of mortality and morbidity worldwide.[1,2] Throughout the last decade improvements in the diagnosis and treatment of atherosclerosis have caused a marked reduction in the morbidity and mortality in men, whereas the rate of recurrent atherothrombotic events, including cardiovascular death, in women has increased.[3,4] Since platelet reactivity plays a pivotal role in thrombus formation and atherosclerosis, dual antiplatelet therapy with both aspirin and clopidogrel has become the cornerstone in the treatment of patients undergoing coronary stent implantation and those presenting with acute coronary syndrome (ACS).[5,6] However, previous studies have suggested that women do not accrue equal therapeutic benefit of antithrombotic therapy.[7,8] Although multiple contributing factors have been described, the physiological mechanism behind this gender disparity remains unclear.[9] Therefore, the aim of the present study is to compare the magnitude of on-treatment platelet reactivity between genders in patients on dual antiplatelet therapy undergoing elective coronary stenting.

METHODS

Population and study design

The **POP**ular-study (*The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI-study)* was a prospective, observational study including consecutive patients with established coronary artery disease scheduled for elective coronary stent implantation. The entry- and exclusion-criteria were described in the original publication. The POPular-study has established that patients exhibiting a high on-treatment platelet reactivity status were at higher risk for adverse events post-PCI.[10]

In the present sub-analysis all patients were on dual antiplatelet therapy with adequate clopidogrel treatment (defined as a maintenance dose of 75 mg daily for >5 days, a loading dose of 300 mg at least 24h before PCI or 600 mg at least 4 hours prior to PCI) and low-dose aspirin of 80-100 mg daily for at least 10 days. Patients were excluded when they had a whole blood platelet count <150.000/μL or used medication (other than aspirin or clopidogrel) knowing to have any effect on platelet reactivity (*i.e.* NSAIDs, dipyramidole, glycoprotein (GP) IIb/IIIa-inhibitors) within one week prior to inclusion. The study was conducted according to the principles of the Declaration of Helsinki and the laws and regulations applicable in the Netherlands. All patients gave written informed consent.

Clinical endpoint

The clinical endpoint was a combination of all-cause death, non-fatal myocardial infarction (defined as the occurrence of ischemic symptoms as well as a spontaneous troponin T value or creatine kinase MB greater than the upper limit of normal), definite stent thrombosis (according to the Academic Research Consortium criteria)[11] and ischemic stroke. An independent committee, blinded for platelet function data, adjudicated all endpoints through review of source documents of medical records.

Blood sampling

Prior to heparinization, whole blood was drawn from the femoral or radial artery sheath. Blood samples were collected into Vacuette® tubes (Greiner Bio-one, Frickenhausen, Germany) containing 3.2% sodium citrate for all platelet function tests. Blood samples for whole blood count were drawn into tubes containing K_3-EDTA. Platelet function testing was performed within 2 hours after blood withdrawal.

Platelet Function Testing

Light Transmittance Aggregometry (LTA)

Light transmittance aggregometry (LTA) was performed using an APACT 4004 aggregometer (LABiTec, Arensburg, Germany) at 37°C. Platelet poor plasma (PPP) was used as a reference for 100% aggregation and maximal platelet aggregation (%) was measured in non-adjusted platelet rich plasma after stimulation with arachidonic acid (AA) in a final concentration of 0.5 mg/ml to determine on-aspirin platelet reactivity and adenosine diphosphate (ADP) in final concentration of 20 µmol/L to determine on-clopidogrel platelet reactivity.

VerifyNow®System

The VerifyNow® (Accumetrics, San Diego, USA) is a whole blood assay designed to measure agonist-induced platelet aggregation. Aspirin induced platelet reactivity was measured with the aspirin assay, which contains arachidonic acid (AA) (1 mmol/L) and clopidogrel response was measured with the P2Y12 assay. This assay contains 20 µmol/L ADP to induce P2Y12-dependent platelet aggregation, and 22 nmol/L prostaglandin E_1 (PGE_1) to minimize the contribution of the ADP-activated P2Y1-receptor to platelet aggregation. Results are described as Aspirin Reaction Units (ARU) and P2Y12 Reaction Units (PRU) respectively.

Statistical analysis

Continuous variables were expressed as mean ± SD, unless otherwise specified, and categorical variables as frequencies (%). All distributions were checked for normality. Differences in continuous variables were compared by independent *t*-test or Mann-Whitney *U* test, as appropriate. Dichotomous variables were compared by chi-square test or Fisher exact test.

Covariate adjustment using a propensity score was performed to reduce confounding factors in the comparison of the magnitude of platelet reactivity between genders. The propensity score was defined as the probability of being a man depending on the baseline characteristics of each patient and used to account for imbalances in the distribution of these characteristics between genders. Prior to calculation of the propensity score, missing data were imputed using the program R. The propensity score for each patient was determined using the following characteristics as covariates in a logistic regression model: clinical characteristics (*i.e.* classic cardiovascular risk factors, previous myocardial infarction (MI), previous percutaneous coronary intervention (PCI) or previous

coronary artery bypass graft surgery (CABG), renal failure, left ventricular ejection fraction <45%), co-medication (*i.e.* use of clopidogrel loading dose and concomitant use of statins, ß-blockers, angiotensin converting enzyme inhibitors, coumarin derivates, calcium channel blocker, proton pump inhibitor, upstream GP IIb/IIIa inhibitor-therapy), laboratory parameters (platelet count, mean platelet volume, white blood cell count, red blood cell count, haemoglobin and hematocrit) and procedural risk factors (*i.e.* total stent length, number of lesions treated, number of stents implanted, bifurcation-stenting, graft-stenting, left anterior descendens coronary artery (LAD), type of stent implanted (bare-metal stent (BMS), drug-eluting stent (DES) or both) and minimal stent diameter). Subsequently, linear regression analysis was performed to compare the magnitude of platelet reactivity between genders, using the propensity scores as a covariate.

To evaluate whether the cut-off value to identify patients at higher risk of atherothrombotic events was similar between genders, a receiver-operator characteristic (ROC) curve analysis was calculated for each test in both genders. The optimal cut-off level was calculated by determining the smallest distance between the ROC-curve and the upper left corner of the graph. To determine whether the cut-off for both genders was similar, a heterogeneity index was calculated. All data were analyzed with SPSS version 17.0 (SPSS, Chicago, IL) and R (version 2.9 http://r-project.org) and a two-sided p-value <0.05 was considered significant.

RESULTS

Study population

A total of 1069 consecutive patients undergoing elective PCI with stent implantation were enrolled, of whom 951 were on aspirin >10 days. The latter comprised the present study population. Due to irregularities in platelet assay supply, as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient. ADP-induced LTA was performed in 936 patients; AA-induced LTA was performed in 925 patients and the VerifyNow P2Y12- Assay in 940 patients. Since the VerifyNow® Aspirin cartridge was started to be used halfway through the POPular-study, this assay was performed in less than half of the population (n=422). Two-hundred-and-thirty-four patients were female (24.6%) and 717 were male (75.4%). Baseline characteristics are depicted in **table 1**. Women were significantly older than men and were more likely to have a familial history of coronary artery disease (CAD). Furthermore, women had a higher platelet count and a lower haemoglobin value.

Gender-specific differences in platelet reactivity

On-aspirin platelet reactivity

Women exhibited a higher magnitude of on-aspirin platelet reactivity as compared to men when measured with LTA (22.4 ± 11.4% vs. 19.8 ± 11.1%, p=0.002). After adjustment for potential confounders, the difference remained significant (22.6 ± 0.8% vs. 20.0 ± 0.9%, p=0.002 [mean ± Standard error of the mean (SEM)]). In contrast, women had a similar magnitude of on-aspirin

Table 1: Baseline characteristics of study population

Clinical parameters	Total population (n=951)	Women (n=234)	Men (n=717)	p-value
Age (yrs)	64 ± 10.6	67 ± 9.9	63 ± 10.6	<0.001
BMI (kg/m2)	27.3 ± 3.9	27.2 ± 4.7	27.3 ± 3.6	0.607
Current smoker	102 (10.7%)	24 (10.3%)	78 (10.9%)	0.785
Diabetes Mellitus	175 (18.4%)	53 (22.6%)	122 (17.0%)	0.053
Hypertension	737 (77.5%)	186 (79.5%)	551 (76.8%)	0.401
Hypercholesterolemia	769 (80.9%)	189 (80.8%)	580 (81.0%)	0.936
Familial history	580 (61.4%)	171 (74.0%)	409 (57.4%)	<0.001
Prior myocardial infarction	432 (45.4%)	94 (40.2%)	338 (47.1%)	0.063
Impaired ejection fraction	133 (14.0%)	35 (15.0%)	98 (13.7%)	0.622
Renal failure	93 (9.8%)	22 (9.4%)	71 (9.9%)	0.809
Medication				
Loading dose clopidogrel	489/951 (51.4%)	116 (51.1%)	373 (53.5%)	0.527
Proton pump inhibitor	270 (29.4%)	70 (31.1%)	200 (28.9%)	0.528
Coumarin derivates	24 (2.5%)	2 (0.9%)	21 (3.0%)	0.073
Calcium channel blocker	365 (39.8%)	91 (40.4%)	274 (39.6%)	0.821
Laboratory Parameters				
Platelet count (x 10^9/L)	273 ± 79	290 ± 78	268 ± 78	<0.001
White Blood Cell count (x 10^9/L)	7.7 ± 2.6	7.9 ± 3.3	7.7 ± 2.3	0.359
Haemoglobin (mmol/L)	8.5 ± 1.0	7.9 ± 0.8	8.7 ± 0.9	<0.001
Procedural Parameters				
Mean no. of stents implanted	1.57	1.55 ± 0.9	1.57 ± 0.8	0.15
Minimal stent diameter (mm)	3.1 ± 0.8	3.1± 1.4	3.1 ± 0.6	0.67
Total stent length (mm)	28.3 ± 17.1	27.4 ± 17.2	28.6± 17.1	0.33
Left Anterior Descending Artery	450/951 (47.3%)	118/234 (50.4%)	**448/713 (46.3%)**	0.27
Bifurcation lesion	32/951 (3.4%)	9/234 (3.8%)	23/717 (3.2%)	0.64
Drug eluting stent	604/946 (63.8%)	156/233 (67.0%)	332/717 (62.8%)	0.26

Definitions
Hypertension: Systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg.
Hypercholesterolemia: A fasting LDL-cholesterol ≥ 3.4 mmol/L or being on statin therapy at the time of inclusion.
Diabetes mellitus: According to the World Health Organization criteria
Family history: One or more first-degree relatives have developed CAD before the age of 55 years (men) or 65 years (women).
Renal insufficiency: Serum creatinine > 120 µmol/L

platelet reactivity using the VerifyNow® Aspirin Assay (437 ± 4.6 vs. 434 ± 5.6, p=0.06 after adjustment [mean ± SEM]). (**Figure 1**)

On-clopidogrel platelet reactivity

The magnitude of on-clopidogrel platelet reactivity was significantly higher in women as compared to men when measured with either LTA (59.6 ± 13.2% vs. 56.9 ± 14.6%, p=0.01) or the VerifyNow® P2Y12 assay (236 ± 73.9 vs. 198 ± 73.9, p<0.0001). All differences remained significant after adjustment for potential confounders in multivariate analysis. (**Figure 1**)

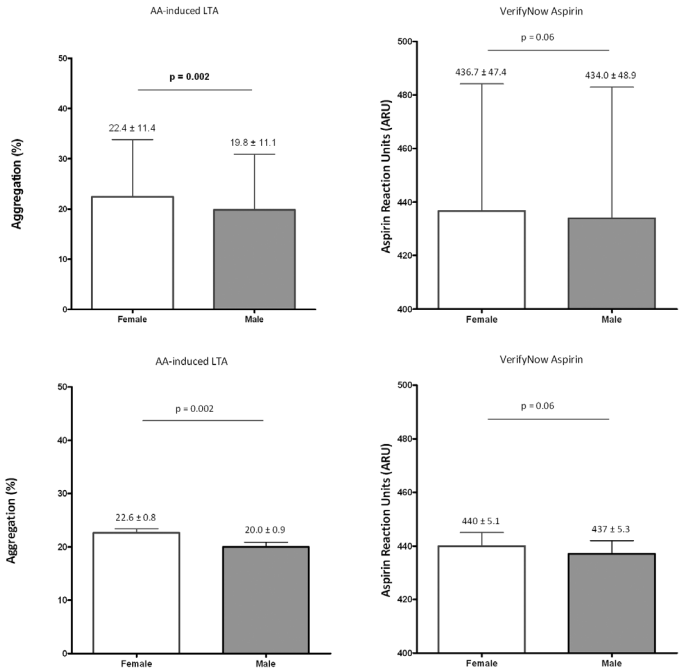

Figure 1: On-aspirin platelet reactivity

The magnitude of on-aspirin platelet reactivity as assessed by AA-induced LTA and the VerifyNow Aspirin-assay prior to (upper part: mean ± SD) and after adjustment for potential confounders (lower part; mean ± SEM).

Gender-specific differences in clinical outcome

Table 2 summarizes one-year clinical outcome. The occurrence of the composite endpoint (62/717 [8.6%] in males vs. 16/234 [6.8%] in females, p=0.58), as well as its single components, was similar between men and women (13/717 [1.8%] men died vs. 3/234 [1.3%] women).

Gender-specific differences in ROC-curve derived cut-off values

Receiver operator characteristic curve (ROC) analysis demonstrated that the cut-off value to identify patients at higher risk of atherothrombotic events was not significantly different between genders (all p-values for heterogeneity>0.10).

Table 2: Clinical outcome

	Female (n=234)	Male (n=717)	OR (95 CI)	p-value
Death, MI, ST, stroke	16 (6.8%)	62 (8.6%)	0.78 (0.43-1.35)	0.58
Death	3 (1.3%)	13 (1.8%)	0.70 (0.13-2.59)	0.47
MI	11 (4.7%)	43 (6.0%)	0.77 (0.35-1.53)	0.35
ST	1 (0.4%)	8 (1.1%)	0.38 (0.01-2.86)	0.36
Stroke	4 (1.7%)	7 (0.8%)	1.76 (0.37-7.01)	0.38

MI = myocardial infarction, ST = Stent thrombosis

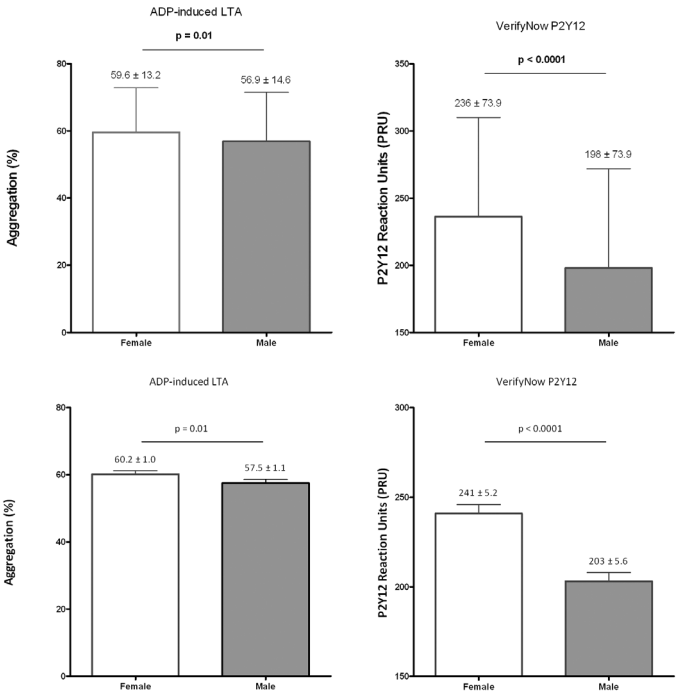

Figure 2: On-clopidogrel platelet reactivity

The magnitude of on-aspirin platelet reactivity as assessed by ADP-induced LTA and the VerifyNow P2Y12-assay prior to (upper part: mean ± SD) and after adjustment for potential confounders (lower part; mean ± SEM).

DISCUSSION

Evidence that gender differences play a role in platelet reactivity, was first reported over 30 years ago[12] and this observation has been confirmed in more recent studies.[13-16] Differences in vessel wall biology between men and women, as well as the direct influence of sex hormones (estrogens, progesterone or androgens) on platelets or their indirect effect on the vasculature might be underlying conditions from a biological point of view.[4,17]

Since platelet reactivity plays a pivotal role in thrombus formation and atherosclerosis, dual antiplatelet therapy with both aspirin and clopidogrel has become the mainstay in the treatment of patients undergoing coronary stent implantation and those presenting with ACS.[5,6,18,19] However,

both drugs result in a wide interindividual range in platelet inhibition[20,21] and the association between high on-treatment platelet reactivity and the occurrence of adverse events is well established.[22-25] As a consequence, identification of particular subgroups of patients with high on-treatment platelet reactivity has gained much attention.[26] Women have been reported to exhibit a higher magnitude of both on-aspirin and on-clopidogrel platelet reactivity more often.[13,27] However, the cause and clinical implication of these findings are uncertain.

The results from the present study support previous findings that women have higher platelet counts[28] and a higher magnitude of on-treatment platelet reactivity than men.[12,13,29] Women exhibited a higher magnitude of on-aspirin platelet reactivity as compared to men using light transmittance aggregometry, but not using the VerifyNow Aspirin-assay. This observation is in line with previous studies reporting a poor correlation between platelet function tests[30,31] and might as well be due to a decreased statistical power, since the VerifyNow aspirin sample was performed in only half of the patient population.[32] The magnitude of on-clopidogrel platelet reactivity was significantly higher in women as compared to men regardless of the test used. In addition, the cut-offs to identify patients at higher risk of atherothrombotic events as well as the prevalence of the primary endpoint were similar between genders. Thus, the present study does not support the hypothesis that higher on-treatment platelet reactivity could account for the gender-differences in clinical outcome and it remains highly questionable whether this gender-related difference in platelet reactivity has clinical relevance.

Previous reported in vitro data suggest that although women have a higher magnitude of platelet reactivity, the response to aspirin is similar or even larger as compared to men.[13,33] This is in line with the observation of a gender-specific meta-analysis on the role of aspirin in primary prevention of cardiovascular disease, demonstrating that aspirin is effective in reducing cardiovascular events in both women and men[7,34]. To date, there are little data on the effects of clopidogrel in women versus men. Whereas conflicting results have been reported on the association between on-clopidogrel platelet reactivity and gender[9], a recent meta-analysis has established that clopidogrel reduces cardiovascular risk in both men and women.[35]

Some issues merit mention. First, the magnitude of platelet reactivity was determined with a single assessment while patients were already on antiplatelet therapy. In this setting, it is impossible to establish whether the difference in platelet reactivity should be attributed to a higher intrinsic (baseline) platelet reactivity in women or to less response. Second, the role of hormonal influences remains unclear, as menopausal status or menstrual cycle has not been assessed in our study but the numbers of premenopausal women in our study are presumably low.

Higher platelet reactivity at baseline among women has been described previously. Although we support the finding that the magnitude of platelet reactivity is higher in women, the absolute difference between genders is small and both the cut-off value to identify patients at risk and the incidence of the composite endpoint was similar between genders. Thus, it is unlikely that the difference in platelet reactivity accounts for a worse prognosis in women.

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Chapter 9

The relationship between platelet reactivity and infarct-related artery patency in patients presenting with a ST-elevation myocardial infarction

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ABSTRACT

Background: Both heightened platelet reactivity and an occluded infarct related artery (IRA) on initial angiography and at the time of primary PCI are associated with a worsened clinical outcome in patients with ST-elevation myocardial infarction (STEMI). However, the relationship between platelet reactivity and IRA patency has not yet been established.

Methods: Consecutive STEMI-patients were enrolled. Patients who had TIMI-flow (thrombolysis in myocardial infarction) 0 or 1 on initial angiography constituted the occluded IRA group and patients having TIMI-flow 2 or 3 comprised the IRA patent group. Platelet function measurements were performed using the PFA-100 COL/ADP cartridge and light transmittance aggregometry without agonist (spontaneous) and after stimulation with adenosine diphosphate (ADP) and arachidonic acid (AA).

Results: Ninety-nine patients were enrolled, of whom 49 presented with an occluded IRA. Multivariate analysis identified the following independent factors to be associated with an occluded IRA; short COL/ADP closure time ($OR_{\text{per quartile increase}}$=0.60; 95% CI, 0.39-.93; *p=0.02*), the 20µmol/L ADP-induced Light transmittance aggregometry ($OR_{\text{per quartile increase}}$ =1.77; 95% CI, 1.15-2.73; *p=0.01* and leukocyte counts (Odds Ratio [OR]=1.21; 95% CI, 1.05-1.39; p = 0.008),).

Conclusions: Heightened platelet reactivity and elevated leukocyte counts are associated with an occluded IRA upon presentation in STEMI-patients. These results emphasize the importance of potent antithrombotic therapy early after the onset of symptoms, to obtain early recanalization of the IRA.

INTRODUCTION

Primary percutaneous coronary intervention (pPCI) with direct recanalization of the infarct-related artery (IRA) is the preferred reperfusion strategy for patients with acute ST-segment elevation myocardial infarction (STEMI).[1-3]

It is well established that spontaneous reperfusion, defined as a patent IRA (open vessel) on initial angiography is associated with higher procedural success (reflected by post-PCI TIMI-flow (thrombolysis in myocardial infarction), myocardial blush grade (MBG)) and more favorable short- and long-term prognosis as compared to patients presenting with an occluded IRA on initial angiography.[4;5] Platelets play a pivotal role in the pathophysiology of STEMI and several studies suggest a relationship between the magnitude of platelet reactivity at the time of PCI and atherothrombotic events post-PCI.[6-9] Thus, both heightened platelet reactivity and an occluded IRA are associated with a worsened clinical outcome in patients with STEMI. However, the relationship between platelet reactivity and IRA patency has not yet been established. The aim of the present study is to determine whether patients with a patent IRA at initial angiography differ from patients with an occluded IRA with respect to the magnitude of platelet reactivity.

METHODS

Study population

In this prospective observational cohort study, consecutive patients undergoing pPCI for acute STEMI were enrolled. STEMI was defined as ST-segment elevation of 0.1 mV in two or more limb leads, or 0.2 mV in two or more contiguous precordial leads of the 12-lead electrocardiogram and with persistent symptoms of chest pain between 30 minutes and 12 hours after symptom onset. Patients were excluded when they presented with haemodynamic instability or shock or if they received thrombolytics or GP IIb/IIIA-receptor inhibitors in the preceding two weeks. According to standard clinical care in the Netherlands, all patients with the diagnosis of STEMI during pre-hospital triage are pre-treated with 500 mg Aspegic® intravenously, a 600 mg loading dose of clopidogrel and 5000 IU of unfractionated heparin in the ambulance prior to transportation. Patients presenting with STEMI at the emergency department were treated with these medications prior to the PCI-procedure at the catheterization laboratory. Upon arrival in the hospital, all patients underwent immediate coronary angiography. All interventions were performed according to current guidelines and the choice of stent type and peri-procedural use of glycoprotein (GP) IIb/IIIa inhibitors was left to the operator's discretion, but the latter were always administered after blood withdrawal. Patients with a whole blood platelet count $<150x10^9$/L were excluded.

Patient characteristics and medical history were obtained by questionnaire. Smoking was identified as any cigarette smoking in the last month. Hypertension was defined as a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg. Diabetes mellitus and hypercholesterolemia were defined according to the World Health Organization criteria.[10] A positive family history was considered when one or more first-degree relatives had developed Coronary Artery Disease before the age of 55 years (men) or 65 years (women).

Blood sampling

Whole blood was drawn from the femoral or radial artery sheath prior to PCI. Blood samples were collected into citrated tubes (3.2% for light transmittance aggregometry and 3.8% for the PFA-100 system) after discarding the first 10 ml of blood. An aliquot of 3 mL blood was collected into a tube containing K_3-EDTA for leukocyte count, platelet count, and mean platelet volume (MPV). These measurements were performed on a routine cell counter (LH 750, Beckman Coulter, Krefeld, Germany).

Platelet function measurement

The magnitude of platelet reactivity was assessed using two platelet functions tests in parallel; the platelet function analyzer (PFA-100) and 'classical' light transmission aggregometry (LTA). A validation of the PFA-100 system demonstrated the coefficient of variation for the test precision was 8.5%[11] and in our laboratory we have established a coefficient of variation of 6.5% using 20 µmol/L ADP. Both platelet function methods were performed between 30 minutes and 2 hours after blood collection. The laboratory technicians were blinded to angiographic data.

Light Transmittance Aggregometry

LTA was quantified in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and maximal (peak) platelet aggregation (%) was measured without using an agonist (spontaneous aggregation) and induced by arachidonic acid (AA 0.5 mg/ml) and adenosinediphosphate (ADP) in a final concentration of 20 µmol/L.

Platelet Function Analyzer-100® System

The Platelet Function Analyzer-100® (PFA-100) System (Dade-Behring - A Siemens company, Germany), measures platelet function, in particular adhesion and aggregation, in whole blood under high shear conditions ($5000s^{-1}$). The time needed to form a platelet plug occluding the aperture cut into a collagen/ADP (COL/ADP)-coated membrane was determined and reported as closure time (CT) in seconds, which is inversely related to the level of platelet reactivity.

Angiographic and ECG analyses

Thrombolysis in myocardial infarction (TIMI) -flow was angiographically determined and classified in 4 grades as described previously.[12] Infarct related artery-patency was assessed on the basis of TIMI-flow; patients with TIMI-flow 0 or 1 on initial angiography constituted the occluded IRA group and patients having TIMI-flow 2 or 3 comprised the IRA patent group.

The extent of residual ST-segment deviation at 1 hr post-PCI was measured at 20 ms after the end of the QRS-complex, using a calliper, as previously described.[13] Two experienced cardiologists, blinded to patient characteristics and platelet function, reviewed all coronary angiograms and ECG's

together. Consensus was achieved in the majority of cases. When no consensus was reached, the expert opinion of a third independent interventional cardiologist was decisive.

Enzymatic infarction size

Creatine kinase (CK) and CK-myocardial band (CK-MB) values were determined at admission and every 6 hr in the first 48 hr after primary PCI. Subsequently these levels were determined every day up to discharge, unless clinical events suggested repeat measurements. The measure for infarction size was peak levels of both CK and CK-MB in plasma.

Statistical analysis

Continuous variables are presented as mean ± SD. Categorical data are reported as frequencies (percentages). Categorical variables were compared using the chi-square test. The distribution of variables was determined by the Kolmogorov-Smirnov goodness-of-fit test. Normally distributed continuous variables were compared with a two-sided unpaired *t* test.

Univariate comparisons of both groups were obtained with the students t-test, the nonparametric Mann-Whitney U test and the Fisher exact test or chi-square test. Logistic regression modelling was used to identify independent correlates of IRA-patency and to adjust for potential confounders such as age, gender, diabetes mellitus, various laboratory parameters, medication (including use of clopidogrel loading dose prior to PCI) and time to PCI. All univariate variables with a p-value <0.10 were included in multivariable analysis (binary logistic regression). All statistical analyses were performed with Statistical Package for the Social Science software, version 14.0 (SPSS Inc, Chicago, IL, USA) and a two-tailed p-value of <0.05 was considered significant.

RESULTS

Study population

Ninety-nine consecutive patients were enrolled, of whom 49 presented with an occluded infarct related artery on initial (pre-intervention) angiography. A total of 99 patients were pretreated with aspirin, 91 with clopidogrel and 94 patients of the population received heparin prior to primary PCI. Nine patients did not receive clopidogrel because of nausea, being intubated or presentation at the emergency department with an uncertain diagnosis, six did not receive heparin and one did not receive aspirin due to an uncertain diagnosis at the emergency department or in the ambulance. These patients received the medications at the catheterization laboratory.

The baseline variables in patients with an occluded IRA (TIMI 0/1 flow) versus an open IRA (TIMI flow 2/3) on initial angiography are summarized in **table 1**, demonstrating the two groups were well balanced. However, patients with an occluded vessel had a significantly higher leukocytes count (12.3±3.6 vs. 10.1±3.3, p<0.01) and a lower blood pressure at admission (127 ± 23.4 vs. 138 ± 20.6, p=0.03).

Table 1 Baseline Characteristics

	Total population (n=99)	Open vessel (n=50)	Occluded vessel (n=49)	P-value
Gender (male)	65/99 (66%)	33/50 (66%)	32/49 (65%)	0.94
Age (yrs)	62.2 ± 13.9	63.6 ± 14.2	60.7 ± 13.5	0.30
BMI (kg/m^2)	25.8 ± 4.0	25.7 ± 4.0	25.9 ± 3.9	0.83
Hypertension	34/92 (37%)	17/47 (36%)	17/45 (38%)	0.87
Hypercholesterolemia	31/83 (37%)	17/40 (42%)	14/43 (33%)	0.35
Diabetes Mellitus	9/96 (9%)	6/50 (12%)	3/46 (7%)	0.31
Current smoker	45/93 (48%)	19/45 (42%)	26/48 (54%)	0.25
Familial history of CAD	46/92 (50%)	23/45 (51%)	23/47 (49%)	0.84
Previous CAD	13/93 (14%)	5/45 (11%)	8/48 (17%)	0.44
Characteristics at admission				
Blood pressure	132 ± 22.6	138 ± 20.6	127 ± 23.4	0.03
Heart rate	76 ± 16	77 ± 17.3	75 ± 16.0	0.65
Ejection fraction	51 ± 8.7	53 ± 7.5	50 ± 9.3	0.16
Killip class I	78/83 (94.0%)	39/41 (95.1%)	39/42 (92.9%)	0.67
Pre-infarction angina	9/88 (10.3%)	4/43 (9.3%)	5/45 (11.1%)	1.00
Pre-hospital clopidogrel	0/99 (0%)	0/50 (0%)	0/49 (0%)	1.00
Laboratory				
MPV in EDTA (fL)	8.7 ± 1.0	8.7 ± 1.0	8.8 ± 1.0	0.67
PLT in EDTA (x10^9/L)	263.0 ± 82.3	256.5 ± 91.1	269.8 ± 72.6	0.43
Inflammatory markers				
CRP (mg/l)	7.8 ± 8.1	7.1 ± 5.8	8.6 ± 9.9	0.38
Leukocytes (x10^9/L)	11.2 ± 3.6	10.1 ± 3.3	12.3 ± 3.6	0.002
Time (minutes)				
Time onset symptoms – cathlab	142.7 ± 91.3	126.3 ± 67.4	158.4 ± 107.8	0.10
Time onset symptoms – medication	114.2 ±93.6	95.2 ± 65.7	133.8 ± 113.2	0.07
Time medication – cathlab	30.5 ± 6.9	32.4 ±18.1	28.4 ± 15.4	0.31

Table 1 : Baseline characteristics

BMI = Body Mass Index; CAD= coronary artery disease; Killip Class (27) 1= individuals with no clinical signs of heart failure; pre-infarction angina(28)=suffering from angina in the 6 months preceding the myocardial infarction; pre-hospital use of clopidogrel= whether patients were on-clopidogrel during the pre-infarction period; MPV= mean platelet volume; PLT: platelet count;

IRA-patency and platelet reactivity

Patients with an occluded IRA at presentation had a significant shorter COL/ADP-CT (98±53sec) as compared to patients with a patent IRA (118±52sec), p<0.01 (**Table 2**). Furthermore, patients with an IRA occlusion had a higher magnitude of 20 µmol/L ADP-induced platelet aggregation as compared to patients with a patent IRA (68.24±11.67% vs. 62.05±16.26%, p=0.03). In contrast, no significant difference could be observed between the two study groups for spontaneous platelet aggregation and AA-induced platelet aggregation (**Table 2**).

Table 2: Platelet reactivity and IRA-patency

Characteristics	Total population (n=100)	Open vessel (n=50)	Total occlusion (n=49)	p-value
LTA spontaneous (% aggregation)	11.4 ± 5.2	11.2 ± 5.2	11.7 ± 5.1	0.69
LTA Arachidonic acid (% aggregation)	27.1 ± 16.8	25.5 ± 14.1	28.7 ± 19.0	0.38
LTA 20 ADP‡ (% aggregation)	65.3 ± 14.4	62.1 ± 16.3	68.3 ± 11.8	0.045
PFA COL/ADP (CT in seconds)	109.0 ± 53.7	118.2 ± 52.4	98.7 ± 54.0	<0.01

Table 2 : Magnitude of platelet reactivity
IRA=infarct related artery; LTA= light transmittance aggregometry; ADP=adenosine diphophate; PFA COL/ADP = Platelet function analyzer using the collagen/adenosine diphosphate cartridge

Binary logistic regression was performed to identify the independent contribution of variables significant associated with IRA patency (**Table 3**). The COL/ADP-CT and 20µmol/L ADP-induced platelet reactivity were divided into quartiles according to the magnitude of platelet reactivity. Quartiles of platelet reactivity based on the COL/ADP-CT were < 72 seconds, 72 to 95 seconds, 95 to 131 seconds and >131 seconds. Quartiles of platelet reactivity as measured with LTA (20 µmol/L ADP) were < 61.0%, 61.0 to 67.3%, 67.3% to 73.5% and >73.5%. After adjusting for factors influencing the magnitude of platelet reactivity, multivariate analysis confirmed the independent prognostic value of 20µmol/L ADP-induced aggregation (odds ratio per quartile increase (OR)=1.77; 95% CI, 1.15-2.73; *p=0.01)* and leukocytes (OR, 1.25; 95% CI, 1.06-1.47; *p*<0.01). The COL/ADP-CT was also an independent predictor for an occluded IRA, indicating that a shorter closure time was associated with a higher probability of a total occlusion ($OR_{\text{per quartile increase}}$=0.60; 95% CI, 0.39-0.93; *p=0.02*). In contrast, no prognostic value was revealed for neither spontaneous nor 0.5 mg/ml AA-induced aggregation.

Table 3: Multivariate analysis

Characteristics	OR	95%-CI	p-value
Leukocytes	1.23	1.07-1.42	<0.01
LTA 20 µmol/L ADP (per quartile)	1.58	1.05-2.39	0.01
PFA COL/ADP (per quartile)	0.62	0.40-0.97	0.04

Table 3 : Multivariate analysis
LTA= light transmittance aggregometry; PFA COL/ADP = Platelet function analyzer using the collagen/adenosine diphosphate cartridge; OR=Odd's Ratio; CI=confidence interval

Table 4

Infarct related artery	Total population (n=99)	Open vessel (n=50)	Occluded vessel (n=49)	P-value
RCA	46/99 (46%)	19/50 (38%)	26/49 (53%)	0.16
LAD	34/99 (34%)	18/50 (36%)	16/49 (34%	0.83
RCX	10/99 (10%)	5 /50 (10%)	5/49 (10%)	1.00
Cumulated ST-deviation 1-hour post PCI	4.1 ± 5.5	3.7 ± 5.0	4.4 ± 6.0	0.61
Residual ST-deviation 1-hour post PCI				0.66
Normalised ST-segment	36/83 (43.4%)	19/39 (48.7%)	17/44 (38.6%)	
1-3 mm	19/83 (22.9%)	7/39 (17.9%)	12/44 (27.3%)	
4-6 mm	11/83 (13.3%)	4/39 (10.3%)	7/44 (15.9%)	
>6 mm	17/83 (20.5%)	9/39 (23.1%)	8/44 (18.2%)	
Magnitude of resolution 1-hour post PCI				0.77
Complete	63/83 (75.9%)	30/39 (76.9%)	33/44 (75.0%)	
Partial	14/83 (16.9%)	7/39 (17.9%)	7/44 (15.9%)	
No	6/83 (7.2%)	2/39 (5.1%)	4/44 (9.0%)	
Myonecrosis markers				
CK	1280.7 ± 212.6	607.9 ± 734.5	1953.5 ± 2385.9	<0.001
CK-MB	190.8 ± 212.6	97.5 ± 83.8	266.0 ± 255.8	<0.001

Table 4: Angiographic and procedural results
RCA=Right Coronary Artery; LAD=Left Anterior Descending Artery; RCX=Circonflex Artery; *Cumulated ST-deviation*[13] the sum of ST-segment deviation in all 12 leads, measured 20 ms after the QRS-complex; *Magnitude of ST-segment resolution*

- complete: >70% resolution
- partial: 30-70% resolution
- no resolution: <30%) resolution

Myonecrosis markers
CK=creatine kinase; CK-MB= creatine kinase myocardial band

IRA patency and clinical outcome

Patients with an occluded IRA had significantly higher values of peak CK (1953.5 ± 2385.9 vs. 607.9 ± 734.5, p<0.001) and peak CK-MB (266.0 ± 255.8 vs. 97.5 ± 83.8, p<0.001). No difference was observed in the residual ST-segment elevation 1-hour post PCI **(Table 4)**.

DISCUSSION

The principal finding of this study is that a heightened platelet reactivity status, represented by a shorter COL/ADP closure time and increased 20 µmol/ADP-induced aggregation was associated with IRA-patency. In contrast, we could not detect a difference between the two groups in spontaneous or AA-induced platelet aggregation. This might be due to the pre-hospital administration of intravenously aspirin, which has an immediate effect on platelet reactivity. In contrast, the loading dose of clopidogrel has not sorted out its full antiplatelet efficacy at the time of blood withdrawal, which is partly caused by the impaired bioavailability of clopidogrel in STEMI-patients.[14-18]

In the present study, elevated leukocyte counts were also found to be associated with an occluded IRA. This is in line with previous reports, suggesting that heightened leukocyte counts might be a marker of a hypercoagulable or thrombotic state.[19-21] The leukocyte response plays a key role in inflammation and it has been reported that an elevation in leukocyte counts is associated with an increased thrombus burden, a less favorable response to thrombolytic therapy and a subsequent worsened clinical outcome.[20]

Multiple studies have demonstrated that spontaneous reperfusion prior to primary PCI is associated with more favorable clinical outcome, reflected by a lower incidence of congestive heart failure and a decreased mortality on the short- and long-term.[4;5] The smaller enzymatic infarction size in patients with a patent IRA as compared to patients with an occluded IRA is in accordance with this observation. In addition, it is well established that a heightened platelet reactivity in STEMI patients is associated with major adverse clinical outcomes (MACE).[8;9] Both Frossard and coworkers as well as Campo and colleagues have demonstrated the association between a longer COL/ADP-CT and a more favorable clinical outcome.[6;7] Since additional antithrombotic agents in STEMI patients prior to pPCI potentially opens the occluded vessel during transportation[22], these findings emphasize the importance of identification of high risk patients[23;24] and early initiation of additional antithrombotic therapy. The Ongoing Tirofiban in Myocardial Evaluation (On-TIME) 2 trial has demonstrated that routine pre-hospital initiation with the use of the GP IIb/IIIa receptor inhibitor tirofiban established a an improved ST-resolution and clinical outcome at 30-days follow up.[13;25;26]

The most important limitation to this study is its observational nature. Therefore, it is impossible to determine whether the relationship between platelet reactivity and IRA patency is causative nor what the underlying mechanisms are. However, the influence of platelets physiology on early coronary patency has been reported previously and the present analysis, describing the fore mentioned relationship in the era of early pre-hospital aspirin and clopidogrel, confirms and strengthens these findings.

In conclusion, the present data suggest that activated platelets and leucocytes play a role in the pathophysiological process leading to IRA occlusion. Because of the superior clinical outcomes associated with an open IRA at initial angiography, combined with the knowledge that additional antithrombotic therapy can achieve coronary reperfusion, these results should encourage the use of more potent antithrombotic therapy early after the onset of symptoms into obtain early recanalization of the IRA.

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Chapter 10

A case-control study on platelet reactivity in patients with coronary stent thrombosis

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ABSTRACT

Background: The pathophysiology of ST has evolved from the identification of single causative factors to a complex multifactorial model.

Objectives: The aim of the present study was to investigate whether patients with a history of stent thrombosis (ST) exhibit a heightened platelet reactivity to clopidogrel and aspirin.

Patients/Methods: Pre- and on-treatment platelet reactivity status to clopidogrel and aspirin, as well as dual antiplatelet therapy resistance was determined in 84 patients with a history of definite ST (cases; 41 early ST, 43 late ST) and in 103 control patients with a previously implanted coronary stent but no ST after the index procedure. Platelet function was evaluated with optical aggregometry, the VerifyNow P2Y12 and Aspirin assays, PFA-100 Innovance P2Y* cartridge, flowcytometric VASP-assay and urine 11-dehydro thromboxane B_2 measurement before and after the administration of a 600mg loading dose of clopidogrel and 100mg aspirin. The study was registered at ClinicalTrials.gov, number NCT01012544.

Results: Patients with a history of early ST clearly demonstrated a higher on-clopidogrel platelet reactivity as compared to controls. Both early and late ST exhibited a heightened on-aspirin platelet reactivity status, and dual antiplatelet therapy resistance was more frequent.

Conclusions: Patients with a history of early ST exhibit a poor response to clopidogrel. Furthermore, both early as well as late ST are strongly and independently associated with a heightened on-aspirin platelet reactivity and dual antiplatelet therapy resistance is more frequent.

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INTRODUCTION

Stent thrombosis (ST) remains the dark site of coronary stenting since it is associated with a considerable morbidity and mortality as well as a high recurrence rate.[1] The pathophysiology of ST has evolved from the identification of single causative factors to a complex multifactorial model.[2] Predictors can be categorized as clinical, procedural, or lesion related. Recently, the involvement of novel determinants has been recognized, including an impaired responsiveness to antiplatelet therapy and a heightened platelet reactivity status despite antiplatelet therapy.[3] Inhibition of circulating platelets with the combined treatment of aspirin and a thienopyridine is currently standard care in preventing ST in patients undergoing coronary stent implantation.[4] However, the wide interindividual variability in the established response to the thienopyridine clopidogrel results in a poor responsiveness in a substantial number of patients.[5] Similarly, aspirin exhibits a certain degree of variability in the residual platelet function among aspirin-treated individuals.[6] Patients exhibiting a high on-treatment platelet reactivity status, either for clopidogrel, aspirin or both, have an increased risk of adverse clinical outcome after coronary stent implantation.[3,7-12]

As ST is a relatively rare complication occurring in 1-5% undergoing coronary stent implantation, prospective studies mostly uncover only few cases of ST during follow-up, and even case-control studies investigating the relation between high on-treatment platelet reactivity and the occurrence of ST have drawn their conclusions on data from relatively small patient cohorts.[13-21] In addition, it is known that platelet reactivity is increased during the early phase after an acute thrombotic event.[22-25] Available studies on the relation between on-treatment platelet reactivity and ST have measured platelet reactivity at the time of the event or shortly thereafter. Hence, the measured high platelet reactivity may have been due to acute phase reaction after ST rather than being a causal factor in the onset of ST.

The aim of the present study was to explore differences in intrinsic and on-clopidogrel and on-aspirin platelet reactivity in a relatively large group of patients with a history of early and late ST, as compared to patients who previously underwent coronary stent implantation but did not incur ST.

METHODS

Study design and Patient population

This study was designed as a single-center case-control study. Cases were patients with a history of an angiographically confirmed ST ('definite' according to the Academic Research Consortium (ARC) criteria[26]). Controls were patients with a previously implanted coronary stent, but no ST during at least 12 months after the index-procedure for clopidogrel-naïve patients (*i.e.* patients not using clopidogrel at the time of inclusion) and at least 3 months for patients on clopidogrel maintenance therapy with 75 mg clopidogrel daily at the time of inclusion (*i.e.* clopidogrel-maintenance group). Control patients were selected from the institutional administrative database that registers all patients undergoing PCI. Because it was deemed unethical to discontinue the clopidogrel therapy in the clopidogrel maintenance group, these cases remained on dual antiplatelet therapy during the

study. All patients were on aspirin at the time of inclusion. Compliance to antiplatelet therapy during ≥14 days prior to inclusion was assessed by interview and verified by pharmacy refill data. Exclusion criteria for both cases and controls were a known allergy to aspirin or clopidogrel, an acute coronary syndrome (ACS) in the past 3 months, recent bleeding diathesis, bleeding disorder, known platelet dysfunction, or an abnormal platelet count (<150x10^9/L). The local institutional Ethics Committee approved the protocol. Written informed consent was obtained from all patients before enrollment. The study was registered at ClinicalTrials.gov, number NCT01012544.

Study Procedure

All eligible cases and controls visited the outpatient clinic for platelet function evaluation, physical examination and a standardized interview. First, blood was drawn for pre-loading platelet function evaluation, and to check for pseudo aspirin resistance due to non-compliance. Then, all subjects received a witnessed 600mg loading dose of clopidogrel and 100 mg aspirin. At 6 hours post-loading, blood was drawn for measurement of on-clopidogrel and on-aspirin platelet reactivity.

Blood and urine sample collection

After an overnight fast and a rest of ≥30 minutes, blood was collected from the antecubital vein into Vacuette® tubes (Greiner Bio-one, Frickenhausen, Germany) containing 3.2% sodium citrate for all platelet function tests, except 3.8% sodium citrate for the PFA-100® system. The first 5 mL of free-flowing blood was discarded to avoid spontaneous platelet activation. Platelet function testing was performed within 2 hours after blood withdrawal. All study participants were asked to provide a first morning urine specimen at the day of inclusion, which were subsequently stored at -80°C until analysis.

Laboratory measurements

Light Transmittance Aggregometry

Light transmittance aggregometry (LTA) was quantified in non-adjusted platelet-rich-plasma (PRP) on an APACT 4004 aggregometer (LABiTec, Arensburg, Germany) and platelet-poor-plasma (PPP) served as the reference for 100% aggregation. The maximal % aggregation was determined after stimulation with either 20 µmol/L adenosine diphosphate (ADP) to measure P2Y12-receptor dependent platelet aggregation, or 0.5 mg/mL arachidonic acid (AA) for on-aspirin platelet reactivity.

The VerifyNow® Aspirin and P2Y12 assays

The VerifyNow® System (Accumetrics, San Diego, USA) is a whole blood assay designed to measure agonist-induced platelet aggregation. The Aspirin assay determines the response to aspirin, using AA in a final concentration of 1 mmol/L as the agonist. Results are reported as aspirin reaction units (ARU). The response to thienopyridines can be measured using the P2Y12 assay that contains 20 µmol/L ADP to induce P2Y12-dependent platelet aggregation, and 22nmol/L prostaglandin E_1

(PGE_1). Results of the P2Y12 assay are reported as P2Y12 reaction units (PRU).[27]

Innovance® PFA P2Y*

The novel PFA-100® analyzer (Dade Behring Marburg GmbH – A Siemens Company, Marburg, Germany) test cartridge Innovance® PFA P2Y* was used, which measures platelet adhesion and aggregation in citrated whole blood under high shear conditions. The membrane of the Innovance® PFA P2Y* cartridge is coated with 20 μg ADP, 5 ng PGE_1 and 125 μg calcium (as calcium chloride). The time needed to form a platelet plug occluding the aperture cut in this cartridge is determined and reported as closure time (CT, seconds).[28]

The flowcytometric vasodilator stimulated phosphoprotein (VASP) assay

Flowcytometric analysis of VASP phosphorylation was performed using a commercially available kit from Biocytex (Marseille, France). Samples were analyzed on a 500 MPL flowcytometer (Beckman Coulter, Marseille, France). The magnitude of platelet activation was expressed as the platelet reactivity index (PRI).

Urinary Thromboxane B_2 measurements

The formation of the platelet-activating TxA_2 from arachidonic acid by cyclooxygenase-1 (COX-1) is inhibited by aspirin. Urinary excretion of the stable TxA_2 metabolite 11-dehydro thromboxane B_2 (11dhTxB_2, pg/mL) was measured using the commercially available kit AspirinWorks® (Corgenix, Westminster, CO, USA). After correction for urine creatinine concentration (mg/dL) results are presented as pg 11dhTxB_2 per mg creatinine.

Definitions

Platelet reactivity was measured before (*pre-loading*) and after clopidogrel loading (*on-treatment*, either on-clopidogrel or on-aspirin platelet reactivity). The occurrence of high on-treatment platelet reactivity was derived from on-treatment platelet reactivity values using a previously defined clinical cut-off as follows: high on-clopidogrel platelet reactivity (HCPR) was 20μmol/L ADP-induced LTA>64,5% or PRU>236[7], high on-aspirin platelet reactivity (HAPR) was AA-induced LTA>20% or ARU>454[29], and dual antiplatelet therapy resistance (DAPR) was the combined presence of HCPR and HAPR.

Statistical analysis

Continuous variables are presented as mean±SD or median [25-75 percentile] in case data deviated from normal distribution, and categorical data as frequencies (%). Differences in continuous variables were compared by independent *t*-test or Mann-Whitney *U* test, as appropriate. Dichotomous variables were compared by χ^2-test or Fisher exact test. Analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc testing or Kruskal-Wallis ANOVA, as appropriate, was

used to compare platelet function test results between controls and cases with early and late ST. Since urinary 11dhTxB2 concentrations were skewed, geometric means±SD were calculated after log transformation of the data to compare results between cases and controls. Multivariate analysis using analysis of covariance (ANCOVA), was used to compare platelet function test results after adjustment for the following factors known to influence platelet function or the occurrence of ST: age, gender, body mass index (BMI, kg/m^2), current smoking, diabetes mellitus (DM), left ventricular ejection fraction (LVEF) <45%, use of proton pump inhibitors (PPI) or calcium channel blockers (CCB)[30], type of stent implanted during the index-procedure (bare-metal stent (BMS), drug-eluting stent (DES) or both), stenting of the left anterior descending artery (LAD) and indication of the index-PCI (ACS or stable angina pectoris [SAP])[2]. Clopidogrel-related analyses were stratified according to clopidogrel treatment at the time of inclusion, *i.e.* clopidogrel naïve group and clopidogrel maintenance group. Analyses of on-aspirin platelet reactivity as well as analyses on the frequency of high on-aspirin (HAPR) and high on-clopidogrel (HCPR) platelet reactivity and dual antiplatelet therapy resistance (DAPR) were performed on the total group. All statistical analyses were performed with SPSS (version 15.0; SPSS Inc., Chicago, IL, USA), and a two-sided *p*-value <0.05 was considered significant.

RESULTS

Patient characteristics

A total of 84 patients with a history of ST were included. Of these, 39 patients (25 with early ST [within 30 days post-PCI] and 14 with late ST [more than 30 days post-PCI]) were clopidogrel-naïve at the time of inclusion, whereas 45 patients (16 with early and 29 with late ST) were on clopidogrel maintenance therapy with 75mg clopidogrel per day. These cases were compared with 74 clopidogrel-naïve controls and 29 controls on clopidogrel maintenance therapy. Further characteristics of the study cohort are illustrated in **Table 1**.

Clopidogrel

I Clopidogrel naïve group

Pre-loading platelet reactivity

Pre-loading platelet reactivity was similar between cases and controls for all platelet function tests (**Figure 1A**).

On-clopidogrel platelet reactivity

After the administration of a 600mg loading dose of clopidogrel, patients with a history of early ST exhibited a higher on-clopidogrel platelet reactivity than controls when platelet reactivity was measured with the VerifyNow P2Y12 assay (250±88 vs. 181±83 PRU, p=0.002), 20 µmol/L ADP-induced LTA (58±16% vs. 46±17%, p=0.005), and the PFA-100 Innovance P2Y* cartridge (300 [80-300] vs. 300 [300-300] sec., p=0.011; **Figure 1B**). These associations remained significant after adjustment

Table 1: Patient characteristics.

	Clopidogrel naive group n=113				Clopidogrel maintenance group n=74			Total group n=187	
Characteristic	Controls n=74	Early ST n=25	Late ST n=14	p-value*	Controls n=29	Early ST n=16	Late ST n=29	p-value*	p-value†
Male gender	59(80%)	23(92%)	12(86%)	0.353	21(72%)	15(94%)	23(79%)	0.233	0.108
Age (years)	60±9.3	63±11.9	61±10.6	0.389	57±11.5	61±11.1	59±8.8	0.545	0.245
BMI (kg/m^2)	28.0±4.2	27.3±4.0	25.9±4.4	0.264	27.1±4.8	26.3±3.4	27.3±4.2	0.736	0.405
Renal insufficiency‡	2(3%)	1(4%)	1(7%)	0.705	1(3%)	0(0%)	2(7%)	0.521	0.441
LVEF ≤45%	8(11%)	10(40%)	5(36%)	*0.002*	3(10%)	4(25%)	3(10%)	0.316	*0.004*
Current smoking	13(18%)	5(20%)	7(50%)	*0.026*	11(38%)	2(13%)	11(38%)	0.157	*0.022*
Family history of CAD	43(58%)	14(56%)	9(64%)	0.877	0(0%)	0(0%)	1(3.4%)	0.566	0.311
Diabetes Mellitus	12(16%)	1(4%)	2(14%)	0.296	4(14%)	3(19%)	7(24%)	0.603	0.368
Hypercholesterolemia	48(65%)	17(68%)	8(57%)	0.791	20(69%)	11(69%)	24(83%)	0.411	0.609
Hypertension	41(55%)	9(36%)	7(50%)	0.245	13(45%)	10(63%)	21(72%)	0.097	0.201
Medication§									
Statin	60(81%)	23(92%)	13(93%)	0.283	27(93%)	14(88%)	26(90%)	0.810	0.473
Coumarin	0(0%)	2(8%)	0(0%)	*0.028*	1(3%)	2(13%)	2(7%)	0.511	*0.041*
Beta-blocker	47(64%)	16(64%)	14(100%)	*0.024*	21(72%)	13(81%)	27(93%)	0.116	*0.001*
CCB	26(35%)	8(32%)	1(7%)	0.115	8(28%)	2(13%)	8(28%)	0.461	0.275
PPI	22(30%)	17(68%)	6(43%)	*0.003*	6(21%)	8(50%)	11(38%)	0.115	0.001
Clinical chemistry									
Platelet count (10^9/L)	239±63	240±53	266±89	0.370	262±80	225±42	256±76	0.231	0.237
Mean platelet volume (fL)	8.6±0.9	8.4±1.1	8.4±0.9	0.746	8.3±0.7	8.4±1.0	8.5±1.0	0.834	0.845

White blood cell count (10^9/L)		6.6±1.7	7.4±2.1	8.1±2.1	*0.011*	7.3±2.1	6.2±2.6	7.6±2.2	0.106	*0.031*
Index-PCI										
LAD stenting		28(38%)	16(64%)	7(50%)	0.079	15(52%)	12(75%)	13(45%)	0.136	*0.015*
Stent type	BMS	31(42%)	16(64%)	7(50%)	0.279	12(41%)	8(50%)	10(34%)	0.355	0.053
	DES	36(49%)	8(32%)	7(50%)	-	15(52%)	8(50%)	19(66%)	-	-
	Mixed	7(9%)	1(4%)	0(0%)	-	2(7%)	0(0%)	0(0%)	-	-
GPIIb/IIIa-blocker		7(9%)	4(16%)	5(36%)	*0.034*	3(10%)	5(31%)	9(29%)	0.119	*0.003*
Time frames[#]										
Index-PCI - inclusion		706 [592-898]	734 [550-1053]	846 [773-1256]	0.195	236 [165-341]	468 [262-743]	1164 [588-1343]	*<0.001*	*<0.001*
Index-PCI – ST		-	2 [0-7]	335 [124-483]	*<0.001*	-	2 [0-6]	461 [258-919]	*<0.001*	*<0.001*
ST – inclusion		-	733 [550-1044]	590 [427-1082]	0.132	-	466 [261-736]	371 [217-663]	0.538	*0.040*

Continuous data are presented as means±SD and statistical analysis of continuous data was performed using univariate ANOVA. Categorical variables as counts (%), with differences between subgroups tested with χ^2.

*p-value for comparison between controls, early ST and late ST within the clopidogrel naive and clopidogrel maintenance groups, and †within the total group. [‡]Glomerular filtration rate (GFR) <60ml/min. [§]Medication at the time of inclusion. [#]Median days [interquartile range], statistical analysis with Kruskal-Wallis analysis of variance. ACE indicates angiotensin converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; BMS, bare metal stent; DES, drug eluting stent; GPIIb/IIIa, glycoprotein IIb/IIIa; LAD, left anterior descending artery; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention.

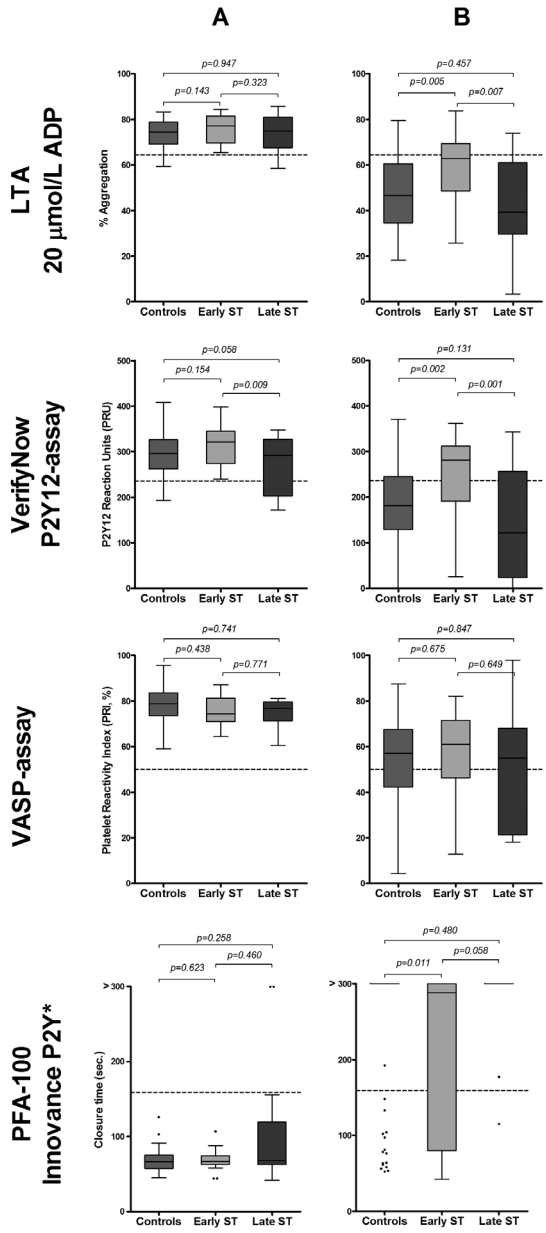


Figure 1: Pre- and on-clopidogrel platelet reactivity. Platelet reactivity was measured using 20 µmol/L ADP-induced LTA, the VerifyNow P2Y12-assay, the VASP-assay and the PFA-100 Innovance P2Y* in the clopidogrel-naïve group, and compared between controls (n=74), early ST (n=25), and late ST subjects (n=14). Panel A: pre-loading platelet reactivity, panel B: on-treatment platelet reactivity. Boxes cover the 25-75 percentiles with horizontal lines indicating medians, and the maximum length of each whisker is 1.5 times the interquartile range. Outliers are depicted as single data points. Horizontal dotted lines indicate cut-offs for high on-clopidogrel platelet reactivity, *i.e.* 64.5% 20 µmol/L ADP-induced LTA, 236 PRU, 50% PRI, and 159 seconds CT for the tests used, respectively. [7, 34]

for potential confounders for LTA (p=0.035) and the VerifyNow P2Y12 assay (p=0.008). There was no difference in the results of the flowcytometric VASP-assay between cases and controls (**Figure 1B**). Furthermore, late ST was not associated with heightened on-clopidogrel platelet reactivity.

II Clopidogrel maintenance group

Pre-loading platelet reactivity

The Innovance P2Y* cartridge revealed a higher pre-loading platelet reactivity in patients with early ST as compared to controls (300 [73-300] vs. 300 [300-300] sec. respectively, p=0.046; **Appendix Figure 1**). No significant differences were observed between controls and patients with early or late ST when pre-loading platelet reactivity was measured with other platelet function tests.

On-clopidogrel platelet reactivity

In patients on clopidogrel maintenance therapy, the administration of a 600 mg clopidogrel loading dose further reduced the magnitude of on-clopidogrel platelet reactivity. A higher on-clopidogrel value of PRU was demonstrated in patients with early ST as compared to controls (PRU 156±107 vs. 91±79 for controls, p=0.026; **Appendix Figure 1B**), while patients with late ST had a higher on-clopidogrel platelet reactivity when measured with the Innovance P2Y* cartridge (300 [300-300] vs. 300 [300-300] sec. respectively, p=0.043; **Appendix Figure 1**). When possible confounders were included in multivariate analysis, these associations were no longer significant. No difference between cases and controls was observed when platelet reactivity was quantified with LTA or the VASP-assay (**Appendix Figure 1B**).

Aspirin

Patients with a history of ST exhibited higher on-aspirin platelet reactivity levels as compared to controls when measured with the VerifyNow aspirin assay (455±63 ARU for early ST vs. 417±57 ARU for controls, p=0.001 [multivariate p=0.002]) and AA-induced optical aggregometry (22±12% for early ST and 20±12% for late ST vs. 14±7% for controls, p<0.001 for both [multivariate p<0.001 and p=0.001, respectively]; **Figure 2**). Furthermore, urinary levels of 11dhTxB_2 were higher in both early ST (1180±250 pg/mg creatinine) and late ST (1362±247 pg/mg creatinine) as compared to controls (891±41 pg/mg creatinine; p=0.047 [multivariate p=ns] and p=0.001 [multivariate p=0.007], respectively).

Dual antiplatelet therapy resistance

DAPR was more prevalent in both patients with early and late ST as compared to controls when assessed by LTA (p=0.003 and p=0.029, respectively) and in early ST when measured with the VerifyNow (p=0.020 compared to controls, **Figure 3**). **Figure 3** shows the distribution of HAPR, HCPR and DAPR stratified by the three patient categories, demonstrating that over 60% of the patients with early ST and 40% of late ST have a high on-treatment platelet reactivity status to either aspirin,

clopidogrel or both, compared to 25-30% of the controls. Furthermore, HCPR occurs more frequent in patients with early ST, while late ST has a higher prevalence of HAPR and DAPR, but less isolated

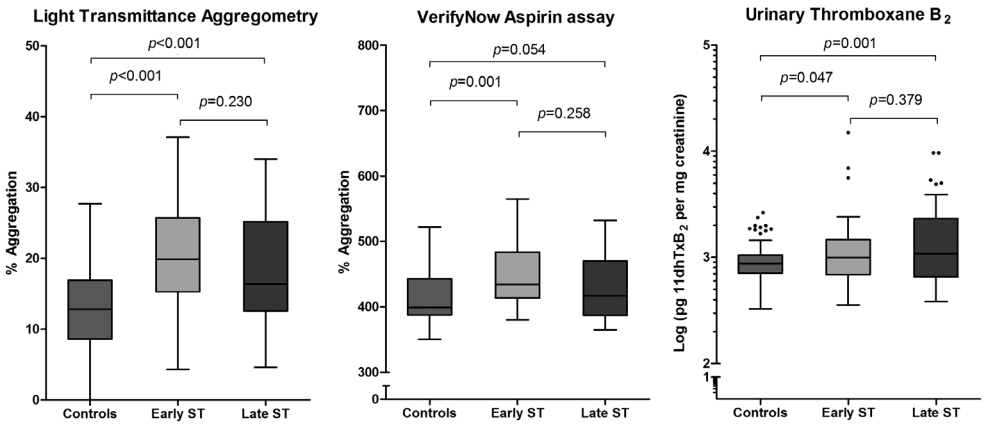


Figure 2: On-aspirin platelet reactivity. Results were compared between controls (n=103) and patients with early ST (n=41) and late ST (n=43). Boxes cover the 25-75 percentiles with horizontal lines indicating medians, and the maximum length of each whisker is 1.5 times the interquartile range. Outliers are depicted as single data points. Since results of the urinary $11dhTxB_2$ measurements were skewed, data are presented on a log-scale and *p*-values represent statistical significance of differences in geometric means.

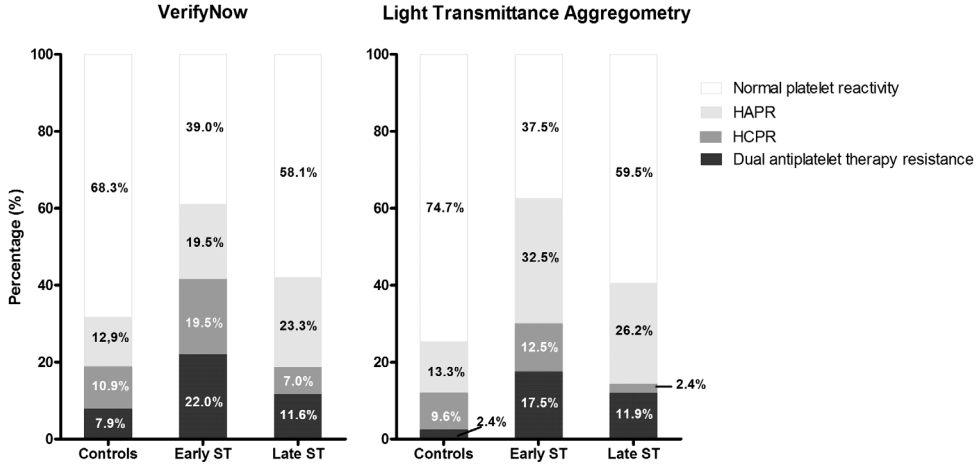


Figure 3: Pattern of normal platelet reactivity, HAPR, HCPR and DAPR. Platelet reactivity was measured using 20 µmol/L ADP- and AA-induced LTA, and the VerifyNow P2Y12- and Aspirin assays. A platelet reactivity value higher than previous determined cut-offs was considered as HCPR (>64.5% LTA or >236 PRU) or HAPR (>20.0% AA-induced LTA or >454 ARU) or DAPR (both HCPR and HAPR). [7, 29] Results were compared between controls (n=103) and patients with early ST (n=41) and late ST (n=43).

HCPR.

DISCUSSION

Throughout recent years, the concept of a high on-treatment platelet reactivity status has been recognized in medical literature as a novel risk-factor for coronary ST and to the best of our knowledge the present study is the largest and most comprehensive evaluation of the relationship between platelet reactivity status and coronary ST.[13-21]

The principle findings of the present study include: *i*) patients with an early ST show an impaired responsiveness to clopidogrel as compared to controls resulting in a high on-clopidogrel platelet reactivity in up to 42% of the cases compared to up to 19% in controls; *ii*) Almost two-third of the patients with early ST exhibit high on-treatment platelet reactivity, to either clopidogrel, aspirin or both; *iii*) patients with a late ST respond well to a 600 mg loading dose of clopidogrel; *iv*) both patients with a history of early and late ST exhibit a heightened on-aspirin platelet reactivity; and v) DAPR is more common in both early and late ST as compared to control subjects.

Previous studies have shown that ST was associated with a heightened on-clopidogrel platelet reactivity. However, platelet function was evaluated early after occurrence of ST.[13-19, 21] Given that myocardial itself is characterized by a transient elevation of platelet reactivity that lasts for at least 30 days, it is unclear whether the established relationship between platelet reactivity and ST was biased by the timing of measurement.[22-25] Therefore, we evaluated platelet reactivity in patients with a history of ST, who were in a stable phase of disease at the time of inclusion. The results of the present study show that patients with ST exhibit a permanently heightened on-treatment platelet reactivity phenotype, implicating a role for genetic factors and/or ongoing disease states.

Patients with late ST had a higher prevalence of HAPR as compared to controls, whereas HCPR was only present as part of DAPR. This finding indicates that an isolated poor response to clopidogrel is less important in the pathophysiology of late ST, which is supported by results from the *Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel – Thrombolysis in Myocardial Infarction (TRITON-TIMI) 38 study*.[31] In this trial it became evident that the prevention of ST by increasing the level of P2Y12-inhibition using prasugrel as compared to clopidogrel, was most prominent for early ST.

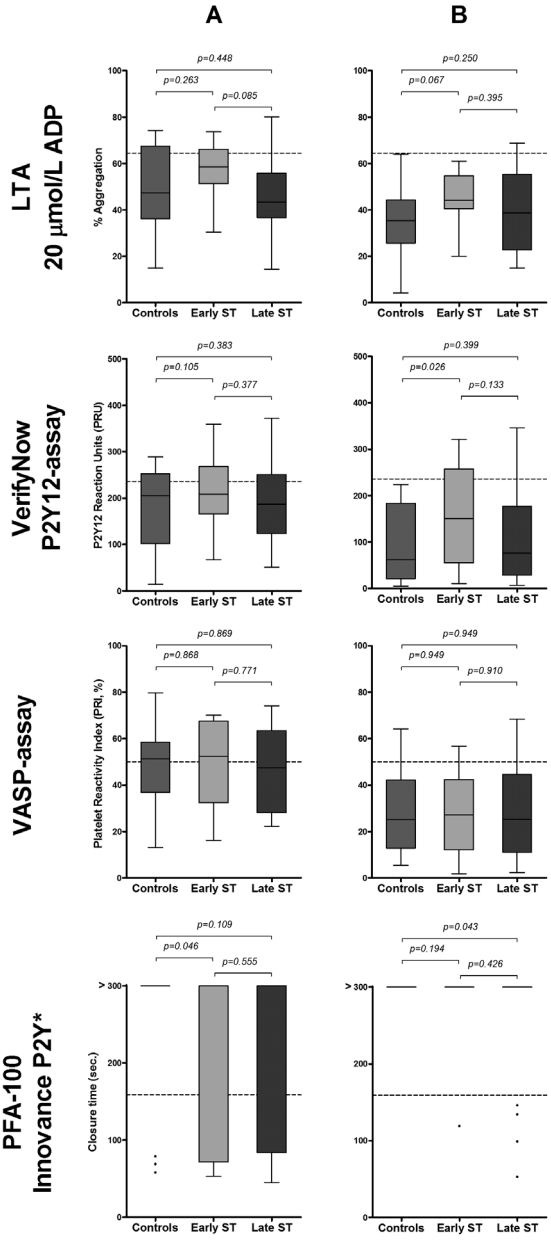
The Innovance P2Y* PFA-100 cartridge became recently available. It was designed to specifically measure the magnitude of P2Y12-receptor inhibition, unlike the Col/ADP-cartridge that appeared to be insufficiently sensitive to the effects of clopidogrel.[28,32] In the present study, the Innovance P2Y* test was able to discriminate between patients with and without a history of early or late ST, in line with the VerifyNow P2Y12 assay and ADP-induced LTA, though this association did not last when clinical variables were included in multivariate analysis.

The flowcytometric VASP-assay has been commonly referred to as 'the biochemical gold standard' for the determination of clopidogrel responsiveness. In the present study, the VASP-assay was not able to detect a difference in response to clopidogrel between patients with and without a history

of ST. These findings are in line with a recent study of Pinto Slottow and colleagues[19], and may be explained by the finding that the VASP-assay was relatively insensitive to lower levels of P2Y12-inhibition, possibly resulting in the incapability to differentiate in the lower regions of the widely ranged response to clopidogrel.[33]

Some aspects of the present study may have hampered the quality of the results and merit attention. Inherent to the study design, an important subgroup of patients was excluded, *i.e.* patients who did not survive the ST or follow-up period. Considering the lethal nature of stent thrombosis, this leaves the evaluation with a substantial risk of survival bias. The obtained results were however in agreement with prospective studies that measured platelet function at the time of the index-procedure, suggesting that the effect of selection bias is small. Furthermore, urinary levels of 11dhTXB$_2$ were measured as an indication of aspirin response, instead of serum thromboxane B$_2$ levels, which is commonly regarded as the 'golden biochemical standard' for detecting aspirin response. Finally, although the total number of patients included in the present study was the largest until present, marked differences between subgroups (*e.g.* early and late ST, clopidogrel naïve and clopidogrel maintenance groups) that were not foreseen *a priori*, required post-hoc subdivision of the total group into smaller subgroups. As a result, multiple comparisons were performed, increasing the likelihood of chance findings.

In conclusion, patients with a history of early ST exhibit a heightened on-clopidogrel platelet reactivity. Furthermore, both early as well as late ST are strongly and independently associated with a heightened on-aspirin platelet reactivity and DAPR is more frequent.



Appendix Figure 1. Pre- and on-clopidogrel platelet reactivity in the clopidogrel-maintenance group. Platelet reactivity was measured using 20 µmol/L ADP-induced LTA, the VerifyNow P2Y12-assay, the VASP-assay and the PFA-100 Innovance P2Y* in the clopidogrel-maintenance group, and compared between controls (n=29), early ST (n=16), and late ST subjects (n=29). Panel A: pre-loading platelet reactivity, panel B: on-treatment platelet reactivity. Boxes cover the 25-75 percentiles with horizontal lines indicating medians, and the maximum length of each whisker is 1.5 times the interquartile range. Outliers are depicted as single data points. Horizontal dotted lines indicate cut-offs for high on-clopidogrel platelet reactivity, *i.e.* 64.5% 20 µmol/L ADP-induced LTA, 236 PRU, 50% PRI, and 159 seconds CT for the tests used, respectively.[7,34]

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Chapter 11

Is platelet inhibition due to thienopyridines increased in elderly patients, in patients with previous stroke and patients with low body weight as a possible explanation of an increased bleeding risk?

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ABSTRACT

Background: The TRITON TIMI-38-study has identified three subgroups of patients having a higher risk of bleeding during treatment with the thienopyridine prasugrel: patients with a history of stroke or transient ischemic attack (TIA), patients ≥75 years, and patients with a body weight < 60 kg. However, the underlying pathobiology leading to this increased bleeding risk remains to be elucidated. The higher bleeding rate may be due to a stronger prasugrel induced inhibition of platelet aggregation in these subgroups. The aim of the present study was to determine whether on-treatment platelet reactivity is lower in these risk subgroups as compared to in other patients in a large cohort on the thienopyridine clopidogrel undergoing elective coronary stenting.

Methods: A total of 1069 consecutive patients were enrolled. On-clopidogrel platelet reactivity was measured in parallel by light transmittance aggregometry (LTA), the VerifyNow® P2Y12 assay and the PFA-100 Collagen/ADP-Cartridge.

Results: Fourteen patients (1069=1.5%) had a prior history of stroke or TIA, 138 patients (14.5%) were older than 75 years and 30 patients (3.2%) had a bodyweight below 60 kilograms. Age ≥ 75 years and a history of stroke were independent predictors of a higher on-treatment platelet reactivity. In contrast, a bodyweight below 60 kilogram was significantly associated with a lower on-treatment platelet reactivity.

Conclusion: In two high-risk subgroups for bleeding, patients ≥75 years and patients with previous stroke, on-clopidogrel platelet reactivity is increased. In contrast, in patients with a low body weight, on-clopidogrel platelet reactivity is decreased, suggesting that only in patients with low body weight a stronger response to a thienopyridine might lead to more bleeds.

INTRODUCTION

Dual antiplatelet therapy with aspirin and the thienopyridine clopidogrel is the therapy of choice in patients undergoing percutaneous coronary intervention (PCI) with stent implantation.[1,2] However, despite this treatment ischemic events still occur and multiple studies have clearly demonstrated a relationship between the magnitude of on-treatment platelet reactivity and the occurrence of atherothrombotic events.[3-8] Therefore, novel antiplatelet agents with more consistent response rates among patients have been introduced. One of these is the thienopyridine prasugrel, which is similarly to clopidogrel a specific, irreversible adenosine diphosphate (ADP)-receptor antagonist but is faster acting and a more potent platelet inhibitor. The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38) demonstrated a significant risk reduction for the occurrence of thrombotic events in patients with an acute coronary syndrome (ACS) undergoing PCI with prasugrel as compared to clopidogrel.[9] This reduction however was counterbalanced by a 30% increased risk of bleeding, suggesting a link between platelet reactivity inhibition and bleedings.[10,11]

Three subgroups of patients were identified as having less clinical efficacy of prasugrel and greater absolute risk of bleeding than the overall cohort. These included (1) patients with a prior history of stroke or transient ischemic attack (TIA), (2) the elderly (≥75 years of age), and (3) patients with a body weight < 60 kg.[9] However, the underlying pathobiology leading to this increased bleeding risk remains to be elucidated.

It has been hypothesized that the higher bleeding rate may be due to a stronger prasugrel induced inhibition of ADP-induced platelet aggregation in these subgroups. Since prasugrel has been introduced only recently, little pharmacodynamic data are available. However, in the POPular-study (The Do Platelet Function Assays Predict Clinical Outcomes in clopidogrel Pretreated patients undergoing elective PCI-study) the influence of the other thienopyridine clopidogrel on the inhibition of platelet reactivity has been determined in patients undergoing elective coronary stent implantation. The aim of the present sub-analysis study was to establish whether the on-clopidogrel platelet reactivity is lower in the three subpopulations at risk for bleeding as compared to in other patients in a large cohort of patients on clopidogrel undergoing elective coronary stenting.

METHODS

Study population

The POPular-study (The Do Platelet Function Assays Predict Clinical Outcomes in clopidogrel Pretreated patients undergoing elective PCI-study) was a prospective, observational study that included consecutive patients with established coronary artery disease scheduled for elective PCI with stent implantation. The entry- and exclusion-criteria were described in the original publication.[3] All patients were on dual antiplatelet therapy with clopidogrel and low-dose aspirin of 80-100 mg daily for at least 10 days, unless they were on long-term treatment with coumarin derivates. This study complied with the Declaration of Helsinki and was approved by the local institutional review

board. Written informed consent was obtained from every patient prior to elective PCI.

Blood sampling and platelet function testing

Prior to heparinization, whole blood was drawn from the femoral or radial artery sheath. After discarding the first 10 ml of blood, samples were collected into citrated tubes (3.2% for light transmittance aggregometry [LTA] and the VerifyNow®-system and 3.8% for PFA). The magnitude of platelet reactivity was measured using three platelet functions tests in parallel; the platelet function analyzer (PFA-100) and 'classical' LTA. All methods were performed between 30 minutes and 2 hours after blood collection.

Light transmittance aggregometry (LTA)

LTA was quantified in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and maximal (peak) platelet aggregation (%) was measured spontaneously and after stimulation by adenosinediphosphate (ADP) in final concentrations of 5 and 20 µmol/L.

The VerifyNow® P2Y12 assay

The VerifyNow® P2Y12 assay (Accumetrics, Inc, San Diego, USA) is an automated whole blood, cartridge-based method to determine the magnitude of platelet agglutination as induced by ADP/prostaglandin E_1.[12] The results are reported in P2Y12 Reaction Units (PRU).

PFA-100

The PFA-100 System (Siemens Healthcare Diagnostics Products GmbH, Germany), measures platelet function, in particular adhesion and aggregation, in whole blood under high shear conditions ($5000s^{-1}$).[13] The time needed to form a platelet plug occluding the aperture cut into a membrane coated with collagen/ADP an agonist was determined and reported as closure time (CT) in seconds, which is inversely related to platelet reactivity. A closure time of >300 seconds was referred to as 'non-closure'.

Statistical analysis

Continuous variables are presented as mean (SD). Categorical data are reported as frequencies (percentages). Categorical variables were compared using the chi-square test or Fisher's exact test when frequencies were <5. The distribution of variables was determined by the Kolmogorov-Smirnov goodness-of-fit test. Normally distributed continuous variables were compared with a two-sided unpaired t test.

Logistic regression modeling was performed to identify independent correlates of the magnitude of platelet reactivity and to adjust for potential confounders. Being part of a high-risk group was entered as a dichotomous variable. All univariate variables with a p-value <0.10 were included in

multivariable analysis (binary logistic regression).

RESULTS

Patient characteristics

A total of 1069 consecutive patients were enrolled, of whom 951 were on aspirin >10 days. The latter comprised the present study population. Owing to irregularities in platelet assay supply, as well as technical failure in a minority of platelet function tests, not all platelet function assays were performed in every patient.

Baseline characteristics of the total population are depicted in **table 1**. Fourteen patients (1.5%) had a history of stroke or TIA, 138 patients (14.5%) were older than 75 years of age and 30 patients (3.2%) had a bodyweight below 60 kilograms. Patients ≥ 75 years were more often female and smoker and had a lower hemoglobin. They were less often treated with statins and the proportion of hypercholesterolemia, renal failure and an impaired ejection fraction was higher. Patients with a history of stroke or TIA more frequently received a loading dose of clopidogrel and more often had a previous history of coronary artery bypass-grafting (CABG). Patient with a bodyweight below 60 kilograms, were significantly older, had lower haemoglobin and were less often treated with statins and beta-blockade. The proportion of females was higher in this group, the frequency of diabetes mellitus and hypertension was lower and the minimal stent-diameter was smaller.

Old age as risk factor for low platelet reactivity

Elderly patients ≥ 75 years, had a significantly higher magnitude of on-treatment platelet reactivity as compared to patients younger than 75 years, regardless of the platelet function test used. (**Figure 1a**, **Table 2**) After adjustment for factors known to influence platelet function (diabetes mellitus, smoking, gender, concomitant use of proton pump inhibitors and the administration of a loading dose of clopidogrel), an age ≥ 75 years remained an independent predictor of a higher magnitude of platelet reactivity, except when 20 µmol/L-induced LTA was used. (**Table 2**)

Cerebrovascular accident as risk factor for low platelet reactivity

In patients with a history of stroke or TIA the magnitude of platelet reactivity was significantly higher as compared to patients without a previous cerebrovascular accident when platelet reactivity was established using LTA (both 5 and 20 µmol/L ADP-induced aggregation). (**Figure 1b**, **Table 2**) After adjustment for potential confounders, a history of stroke or TIA remained an independent predictor of a higher level of aggregation. In contrast, no significant difference was found between the group with and without a history of stroke or TIA when platelet reactivity was assessed using the VerifyNow® P2Y12 assay or the PFA COL/ADP-cartridge. (**Table 2**)

Low bodyweight as risk factor for low platelet reactivity

Aggregation as measured by 20 µmol/L ADP-induced LTA was significantly lower in patients with a

Table 1: baseline characteristics

Clinical parameters	Total Population
Age (yrs)	64 ± 10.6
BMI (kg/m2)	27.3 ± 3.9
Gender (male)	717/951 (75.4%)
Hypertension	737/951 (77.5%)
Hypercholesterolemia	769/951 (80.9%)
Diabetes Mellitus	175/951 (18.4%)
Family History	580/951 (61.0%)
Current smoking	107/951 (11.3%)
Impaired ejection fraction	133/951 (14.0%)
Renal insufficiency	72/951 (7.6%)
Prior myocardial infarction	519/951 (54.6%)
Prior PCI	304/951 (32.0%)
Prior CABG	93/951 (9.8%)
Medication	
Loading dose clopidogrel	489/951 (51.4%)
Statin	767/951 (80.7%)
Beta-blocker	733/951 (77.1%)
ACE-inhibitor	345/951 (36.3%)
Proton pump inhibitor	270/951 (28.4%)
Calcium Channel Blocker	365/951 (38.4%)
Oral antidiabetics	62/951 (6.5%)
Coumarin derivates	24/951 (2.5%)
Laboratory Parameters	
Platelet count ($x10^9$)	273.4 ± 78.8
White Bloodcell Count ($x10^9$)	7.7 ± 2.3
Hemoglobin (mmol/L)	8.6 ± 2.2
Procedural Parameters	
No.of stents implanted	1489/950 1.57
No.of lesions treated	1317/951 1.38
Minimal Stent diameter (mm)	3.1 ± 0.8
Total Stent length (mm)	28.3 ± 17.1
Bifurcation lesion	32/951 (3.4%)
Drug eluting stent	604/946 (63.8%)
Left anterior descending artery	450/951 (47.3%)
Graft	28/951 (2.9%)

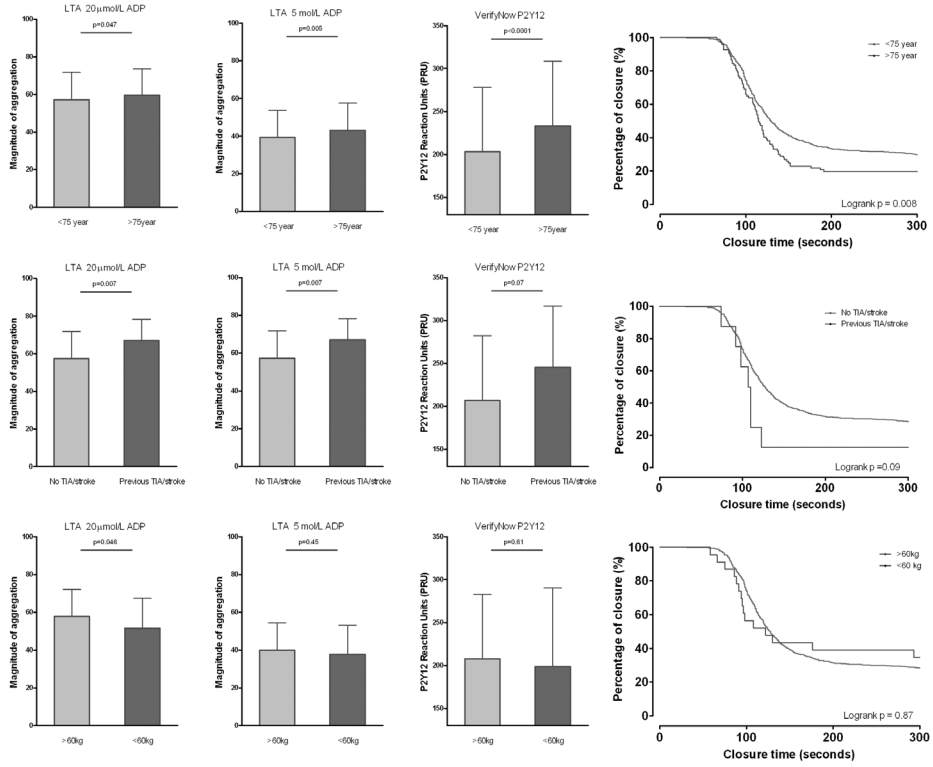

Figure 1: Magnitude of platelet reactivity

Magnitude of platelet reactivity according to the three tests used.

Since the PFA-100® System confines detection of a closure time to a 300-s window, the results of the PFA-100® System are depicted as a cumulative Kaplan Meier time-to-aperture-closure plot and a log-rank test was used.

A) In patients <75 years vs. patients ≥75 years of age
B) In patients with a history of TIA or stroke vs. patients out history of TIA or stroke
C) In patients <60 kg vs. patients ≥60 kg

bodyweight below 60 kilograms as compared to patients with a higher bodyweight. None of the other tests identified significant differences between patients with a low bodyweight and patients with a bodyweight over 60 kilogram. (**Figure 1c**, **Table 2**) After adjustment for potential confounding factors, lower body weight remained significantly associated with an intensified platelet response to clopidogrel as established by either 20 µmol/L ADP-induced LTA and became significant when measured with the VerifyNow® P2Y12 assay. (**Table 2**)

Table 2: Magnitude of platelet reactivity

Magnitude of platelet reactivity in the elderly

	<75 yr (n=815)	≥75 yr (n=136)	p value	After adjustment	
				Difference	p value
LTA 20 ADP	57.2 ± 14.5	59.7 ± 13.8	0.047	2.10	0.11
LTA 5 ADP	39.3 ± 14.5	43.1 ± 14.4	0.0046	3.3	0.01
PRU	202.9 ± 74.9	233.5 ± 75.2	<0.0001	25.9	0.0001
PFA COL/ADP	NA	NA	0.008	NA	0.008

Magnitude of platelet reactivity in patients with a history of a cerebrovascular event

	No TIA/stroke (n=937)	Previous TIA/stroke (n=14)	p value	After adjustment	
				Difference	p value
LTA 20 ADP	57.4 ± 14.4	67.0 ± 11.3	0.007	8.46	0.03
LTA 5 ADP	39.7 ± 14. 6	48.8 ± 10. 6	0.007	8.46	0.03
PRU	206.8 ± 75.6	245.5 ± 71. 5	0.07	37.4	0.06
PFA COL/ADP	NA	NA	0.09	NA	0.11

Magnitude of platelet reactivity in patients with a low bodyweight

	≥60 kg (n=921)	<60kg (n=30)	p value	After adjustment	
				Difference	p value
LTA 20 ADP	57.7 ± 14.3	51.6 ± 15.8	0.046	-7.2	0.007
LTA 5 ADP	39.9 ± 14.5	37.7 ± 15.4	0.45	-3.9	0.16
PRU	207.7 ± 75.1	198.7 ± 91.4	0.61	-28.1	0.04
PFA COL/ADP	NA	NA	0.87	NA	0.76

LTA=Light transmittance aggregometry, ADP=Adenosine diphosphate, PRU=P2Y12 reaction units, PFA COL/ADP= Platelet function analyzer using the collagen/ADP-cartrdige

DISCUSSION

Whereas TRITON TIMI-38 demonstrated that prasugrel, a thienopyridine resulting in lower on-treatment platelet reactivity as compared to clopidogrel, was associated with less recurrent atherothrombotic events in ACS patients undergoing PCI, an increased risk of bleeding was observed in patients treated with prasugrel.[9] The presence of a therapeutic window was already acknowledged by Paracelsus, who stated as early as in the 15th century that "*All drugs are poisons, the benefit depends on the dosage*".[14] There is currently a growing body of evidence supporting the association between bleeding and adverse outcomes, including myocardial infarction, stroke and death.[15-17] and several studies have suggested a link between the inhibition of platelet reactivity and the occurrence of bleeding.[10,18-20] Thus, the identification of a window of platelet inhibition that on the one hand prevents atherothrombotic events and on the other hand does not lead to an increase in bleeding events, is of utmost importance.[21]

The TRITON TIMI-38-study has identified three subgroups of patients having a higher risk of bleeding during treatment with prasugrel: 1) patients with a prior history of stroke or transient ischemic attack (TIA), 2) the elderly (>75 years of age), and 3) patients with a body weight less than 60 kg.[9] However, the underlying pathobiology leading to this increased bleeding risk remains to be elucidated. It has been hypothesized that the higher bleeding rate might be the consequence of a stronger prasugrel induced inhibition of ADP-induced platelet aggregation in these subgroups. Since prasugrel has been introduced only recently, little pharmacodynamic data are available. The present study, with the aim to determine whether on-clopidogrel platelet reactivity is lower in these risk subgroups as compared to in other patients, demonstrated that in the two high-risk subgroups for bleeding, patients > 75 years of age and patients with previous stroke, on-treatment platelet reactivity is contrarily increased. However, in the third high-risk subgroup for bleeding, patients with a low body weight, on-treatment platelet reactivity is indeed decreased. When these data are applied to the hypothesis that prasugrel leads to a stronger platelet inhibition, it seems that only in patients with low body weight a stronger response to prasugrel might have led to more bleeds in TRITON TIMI 38.

These observations are in line with results from a recent analysis of 16 phase-I clinical pharmacological studies performed in healthy patients. In this analysis no effect of advanced age on the availability of the active metabolite of prasugrel was perceived.[22] On the contrary, in the TRITON-TIMI 38, patients ≥ 75 years had 19% higher exposure to the active metabolite as compared to those <75 years and even 25% higher exposure as compared to patients <60 years of age.[23] However, in the latter the concentration of the active metabolite was not measured, but estimated from its inactive metabolite. In contrast, bodyweight had the greatest influence on exposure to the active metabolite of prasugrel in both clinical pharmacology studies and the TRITON-TIME 38, with an increase in exposure as bodyweight decreased. Exposure was 40% higher in individuals <60 kg as compared to those ≥ 60 kg.[22,23] Modelling data suggest that decreasing the maintenance dose of prasugrel to 5 mg in these subjects would reduce exposure to the active metabolite to levels consistent with those <75 years and ≥ 60 kg.[22] Both European and American regulatory agents therefore recommend a daily dose of 5 mg in patients<60kg. For patients ≥ 75 years, the US Food and Drug Administration (FDA) advices that prasugrel is generally not recommended, but might be considered in patients at high risk of recurrent atherothrombotic events at a maintenance dose of 10 mg in those ≥ 60 kg.[23] On the contrary, the European Medicines Agency recommends prasugrel to be avoided in the elderly, but if used, the dose should be halved to 5 mg.[23] Taken the findings from the present study into account and the fact that elderly have a higher risk of bleeding as compared to younger individuals, even when not on-thienopyridine therapy,[24] we do not consider prasugrel to result in an excess in platelet inhibition thereby accounting for the increased bleeding risk in this population.

In conclusion, the results from the present analysis confirmed previous studies and identified body weight as the most influential covariate on the magnitude of ADP-induced platelet reactivity, which might have implications for prasugrel maintenance dose in daily clinical practice.

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Chapter 12

Discussion

New options in antiplatelet therapy: Navigating between Scylla and Charybdis

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INTRODUCTION

Dual antiplatelet therapy with aspirin and a thienopyridine is the mainstay in the treatment of patients with acute coronary syndrome (ACS) and those undergoing PCI with stent-implantation.[1,2] However, clopidogrel has a number of disadvantages, the most important one being the highly variable magnitude of on-treatment platelet reactivity.[3] Consequently, the search for and validation of new antiplatelet agents have been undertaken with increasing eagerness. This has led to the development of new P2Y12 antagonists, which are in various phases of investigation (**Table 1**).

Table 1 Novel P2Y12-antagonists

Medicijn	Structuur	Direct/ indirect	Reversible	Route	Frequency	Phase
Ticlopidine	Thienopyridine	Indirect	Irreversible	Oral	Bidaily	Approved by FDA and EMEA
Clopidogrel	Thienopyridine	Indirect	Irreversible	Oral	Once daily	Approved by FDA and EMEA
Prasugrel	Thienopyridine	Indirect	Irreversible	Oral	Once daily	Approved by FDA and EMEA
Ticagrelor	Cyclo-pento-triazolo-pyrimidine	Direct	Reversible	Oral	Bidaily	Approved by EMEA
Cangrelor	ATP-analog	Direct	Reversible	Intra-venously	Infusion	Phase-III
Elinogrel		Direct	Reversible	Oral/ intra-venously	Infusion, bidaily afterwards	Phase-II

Clopidogrel

Clopidogrel (Plavix) is a prodrug that requires conversion by hepatic P450 isoenzymes to its active metabolite. Most of the clopidogrel (85%) is hydrolyzed by carboxylase to an inactive carboxylic acid metabolite, whereas the remaining 15% is transformed rapidly into its active metabolite that is able to exert its antiplatelet response by irreversibly inhibiting the binding of adenosinediphosphate (ADP) to the P2Y12 receptor.[4-6] Recently, paraoxonase-1 (PON1) was identified as the crucial enzyme in clopidogrel bioactivation.[7] Although the clinical benefit of clopidogrel is well established, the optimal dosing is subject of intense debate.[2,8]

Dosing

As compared to the standard dose of clopidogrel used in early trials, more recent studies have shown that higher doses of clopidogrel lead to greater, more rapid, and more consistent platelet inhibition. A meta-analysis, consisting of ten studies, including a total of 1567 ACS-patients, demonstrated that a loading dose varying between 450-900 mg was more effective in reducing death and myocardial infarction post-PCI (OR = 0.54, CI 0.32-0.92, p=0.02), without an increase in bleeding. Recently, the CURRENT-OASIS-7-trial randomized 25 086 ACS patients referred for an invasive strategy to either

high-dose or standard-dose clopidogrel.[9] The high-dose clopidogrel group received a 600-mg loading dose and then 150 mg once daily for next seven days, followed by 75 mg once daily until 30 days. Patients in the standard-dose clopidogrel arm received a 300-mg loading dose, followed by 75 mg once daily until 30 days. The primary endpoint, a composite of cardiovascular mortality, myocardial infarction and cerebrovascular accident (CVA) at 30-days did not differ between both treatment arms, but an increase in bleeding with the higher clopidogrel dose was observed. The pre-specified analysis of the subpopulation undergoing PCI (n=17.2631, 95% stent-implantation) showed a significant reduction in definite stent thrombosis with the higher clopidogrel loading dose, at the cost of an increase in major bleeding.[10]

So, the choice for a higher loading dose in patients with ACS or those undergoing an early intervention is not only recommended by the guidelines, but is also advocated by the results of the CURRENT-OASIS-7-trial. On the contrary, in individuals who have planned conservative treatment or in whom invasive assessment might be delayed beyond 72 hours, the standard loading dose of clopidogrel should be used. The debate on the maintenance dose is still ongoing. In our opinion, patients with a low risk of bleeding and a high chance of an early percutaneous intervention should receive a loading dose of 600 mg of clopidogrel, followed by 150 mg for one week and 75 mg thereafter (for at least one year). In all other cases, we consider the small benefit of the higher dose counterbalanced by the higher bleeding risk and we advise to use the standard loading dose of 300 mg followed by a maintenance dose of 75 mg.

High on-treatment platelet reactivity

Thus, a higher dose of clopidogrel appears to reduce a part of the problems in ACS patients undergoing coronary stent implantation. Despite this higher dose, a large interindividual variability in the magnitude of platelet inhibition remains.[11,12] There is growing evidence that the magnitude of platelet reactivity, while treated with clopidogrel, is associated with worse clinical outcome.[13-15] Multiple factors influencing the efficacy of clopidogrel have been identified (**Table 2**), among others a lower biological availability of the active metabolite of clopidogrel has been demonstrated.[16-18] This can be the result of a genotype with a reduced activity of the enzyme as well as interaction with medication that is metabolized by the hepatic CYP-enzyme-system, thereby reducing the efficacy of clopidogrel.[19-21]

A study on the influence of proton pump inhibitors on the pharmacokinetics of clopidogrel demonstrated an interaction between clopidogrel and omeprazol, but did not establish an interaction between clopidogrel and pantoprazol.[22] This observation urged the US Food Drug Administration (FDA) as well as the European Medicines Agency (EMEA) to give off a black box warning for concomitant use of clopidogrel and (es)omeprazol.[23] However, the only randomized study thus far did not identify any effect of omeprazol on clinical endpoints and, more importantly, showed a significant reduction in the number of gastro-intestinal bleedings.[24] Combining these data with the observation of a new study suggesting the interaction between omeprazol and clopidogrel

Table 2: Factors influencing the efficacy of clopidogrel

Pharmacological factors	
	Inadequate dosing of clopidogrel
	Non-compliance
	Drug-drug interaction: *statins, proton pump inhibitors calciumantagonists*
	Magnitude of intestinal absorption
	Magnitude of excretion
	Metabolism (high *BMI)*
Genetic factors	
	Polymorphisms in the hepatic CYP -enzymesystem
	Polymorphisms in the P2Y12-receptor
	Polymorphisms in other platelet receptors: *α_2-adrenerg receptor, P2Y1-receptor, glycoproteïne IIb/IIIa-receptor*
Platelet related factors	
	Loading dose and time to platelte function testing
	Increased baseline (intrinsic) platelet reactivity: *Clinical presentation :* (myocardial infarction, (excessive) exercise, inflammation) *Patient related factors* (diabetes mellitus, renal failure, smoking)
	Increased turnover
	Increased exposure to ADP
	Increased P2Y12-receptor-activation
	Choice of anticoagulant used in platelet function assay

CYP-enzymsystem= hepatic cytochrome P450-enzymesystem, BMI= body mas index, ADP = adenosine diphosphate

is restricted to the maintenance dose of clopidogrel and proposing the idea that a higher loading dose might overcome the interaction[25], we do not consider the cessation of proton pump inhibitors in patients onclopidogrel a necessity.

Moreover, the variability in response to clopidogrel is determined by pharmacological as well as genetic factors. Four studies have simultaneously established that polymorphisms of the CYP-enzyme-system, *CYP2C19*-genotypes with a loss-of-function allele in particular, are associated with a lower biological availability of the active metabolite of clopidogrel and consequently a lower inhibition of platelet reactivity.[26-29] In patients with ACS, these loss-of-function alleles led to a reduced efficacy of clopidogrel, with a higher risk of cardiovascular complications. These findings have recently urged the FDA to change the information on the prescription of clopidogrel. The FDA recognizes the reduced efficacy of clopidogrel and advices the use of genetic tests to establish the genotype and to consider other medication or doses in those carrying a loss of function allele.

To overcome the disadvantages of clopidogrel, novel P2Y12 antagonists have been developed, which are in various phases of investigation (**Table 1**) to determine whether they can result in better, more rapid, or both, antithrombotic effects than clopidogrel, without an unacceptable increase in

hemorrhagic or other side effects. In contrast to clopidogrel, these new options are hardly affected by the loss-of-function CYP2C19-allele or interaction with concomittant medication (**Table 1**).

Prasugrel

Prasugrel (Efient) is an orally administered thienopyridine, that like clopidogrel needs to be metabolized in the liver. The active metabolite of prasugrel is as effective as the active metabolite of clopidogrel in irreversibly blocking the P2Y12-receptor, but the in-vivo generation of the active metabolite of prasugrel is much more efficient and therefore a higher concentration of the active metabolite is available. (**Figure 1**) As a consequence prasugrel inhibits ADP-induced platelet aggregation more rapidly, more consistently, and to a greater extent than do both the standard and higher doses of clopidogrel[30]. Furthermore, the recently published SWAP (SWitching Anti Platelet)

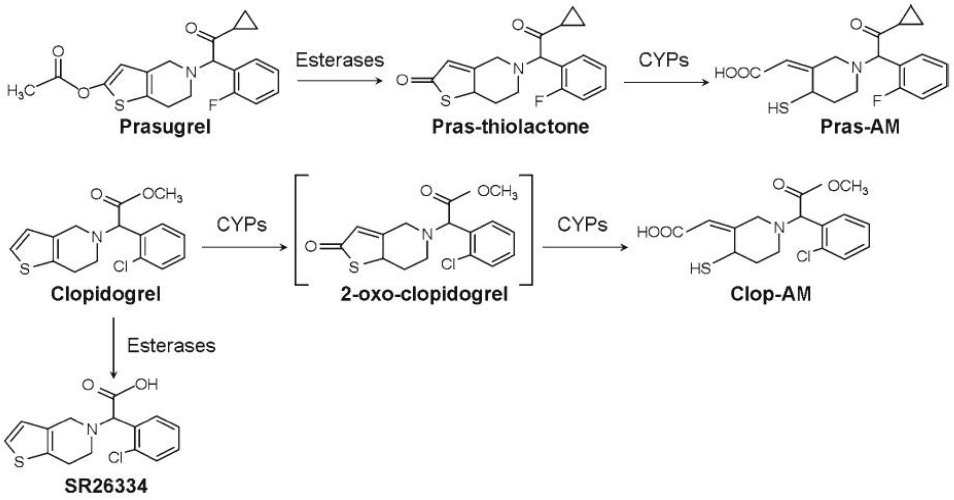

Figure 1: Metabolism thienopyridines.
Schematic representation of the metabolism of both clopidogrel and prasugrel

Study demonstrated a further reduction in platelet reactivity using prasugrel in patients with ACS who were adequately treated with clopidogrel.[31]

The clinical efficacy of more potent platelet inhibition using prasgrel was established in the TRITON-TIMI 38 (TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet inhibitioN with prasugrel- Thrombolysis In Myocardial Infarction 38).[32] In this study, randomizing 13.608 patients with ACS undergoing elective PCI to either prasugrel (60mg loading dose followed by 10mg maintenance dose) or clopidogrel (300mg followed by 75mg maintenance) prasugrel was associated with a significantly reduced rate of ischemic events, including stent thrombosis, but with an increased risk of bleeding, including fatal bleeding. In two subpopulations, patients with STEMI and patients suffering from diabetes mellitus,[33,34] the advantage of prasugrel over clopidogrel was

even more pronounced, showing a reduction in the number of thrombotic complications without an increase in bleeding events. A post-hoc analysis identified three subgroups of patients that had less clinical efficacy and greater absolute levels of bleeding than the overall cohort, resulting in less net clinical benefit or in clinical harm. These subgroups comprise patients with a history of TIA or stroke, patients older than 75 years and patients with a body weight below 60 kilograms. This observation suggests that the more potent platelet inhibition caused by prasugrel might not be indicated for everyone. At this moment we consider prasugrel indicated in patients presenting with a stent thrombosis while on-clopidogrel, patients undergoing a primary PCI for STEMI and patients with DM, in line with the European guidelines as well as the British National Institute for Health and Clinical Excellence. In an elective PCI-setting, we are more reserved and advise to estimate the risk based on clinical characteristics and the magnitude of platelet recativity (**Figure 2**).

Figure 2: POPular-risk score

POPular Risk Score

1 punt	Genetisch: CYP2C19 *1/ *2
2 punt	Genetisch: CYP2C19 *2/ *2
2 punt	Hoge plaatjesreactiviteit (PRU≥236)
½ punt	Diabetes
½ punt	Stent lengte > 30 mm
½ punt	LV Ejectie Fractie < 30 %
< 2 punt	Clopidogrel
≥ 2 punt	Prasugrel

Ticagrelor

Ticagrelor (AZD6140) is not a thienopyridine but a cyclo-pentyl-triazolo-pyrimidine, a direct P2Y12 antagonist that does not require metabolism of a prodrug . Similar to prasugrel, in phase-2-studies ticagrelor resulted in a faster onset and a more potent platelet inhibition as compared to clopidogrel.[35] Considering the short half time and the reversibility of the receptor-binding, ticagrelor needs to be administered twice daily. The clinical efficacy of ticagrelor was investigated in the PLATO (Platelet Inhibition and Patient Outcomes)-study, comparing ticagrelor (180 mg/ 90 mg bidaily) to clopidogrel (300 to 600 mg bolus/75 mg maintenance dose) in 18.624 patients admitted with ACS, with or without ST-elevation. Ticagrelor was more effective than clopidogrel in reducing the primary endpoint, combining cardiovascular death, myocardial infarction or stroke (HR=0.84 (0.77-0.92), p=<0.001) without an increase in the number of major bleeding, but with an increase in the rate of non-CABG related bleeding.[36] The EMEA has approved the use of ticagrelor, but the FDA has delayed her decision on the approval of ticagrelor. Considering the results, the indication

of ticagrelore will be comparable to that of prasugrel although ticagrelor appears to be indicated in all-comers, whereas prasugrel might remain reserved to those undergoing PCI.

Under development

Cangrelor is a direct-acting, reversible P2Y12-receptor antagonist, which in contrast to the forementioned orally administered P2Y12-antagonists, is administred intravenously. In phase II-studies it was oberved that cangrelor resulted in a rapid onset and consistent higher level of platelet inhibition as compared to clopidogrel, without an increase in the rate of bleeding. There is a rapid reversal of cangrelor's effect after the end of the infusion (<1 hour). Recently two phase-III-studies were presented in patients with undergoing PCI; the CHAMPION-PCI (Cangrelor versus standard therapy to achieve optimal management of platelet inhibition)[37] and the CHAMPION-PLATFORM[38]. The two trials were similar in that they both included predominantly ACS patients for whom a strategy of deferred ADP-receptor blockade had been chosen (ie, the drugs were given after diagnostic angiography had established the indication for PCI). They differed, however, in the timing of clopidogrel administration. In the CHAMPION-PCI trial, patients were randomized to cangrelor infusion (started within 30 minutes before PCI and continued for two hours) or clopidogrel 600 mg orally, again given within 30 minutes before PCI. After the infusion, patients in the cangrelor group were then given clopidogrel 600 mg, and patients who had already received clopidogrel were given placebo tablets. In the CHAMPION-PLATFORM trial, the same two-hour cangrelor infusion was compared with a placebo infusion. All patients then received 600 mg of clopidogrel. In the cangrelor group, clopidogrel was given at the end of the infusion, and in the placebo group, clopidogrel was given at the end of the PCI procedure. Both studies were stopped prematurely, after an interim analysis indicated that they would be unlikely to show benefit for the primary end point. So, the CHAMPION-PCI was testing cangrelor vs clopidogrel with both being given up front, whereas the CHAMPION-PLATFORM was testing cangrelor up front vs delayed clopidogrel. Both trials failed to show a significant benefit of cangrelor on the primary end point, a combination of death, myocardial infarction and ischemia-driven revascularization, both at short (48 hours) as well as longer term (30 days).

Elinogrel (PRT060128, Novartis, Basel, Zwitserland) is a direct-acting, reversible P2Y12- antagonist with a novel structure and can be administered both intravenously as well as orally. Recently, the first results from a phase-II study have become available. The INNOVATE-PCI,[39] presented at the annual scientific sessions of the ESC, evaluated the safety and the tolerability of elinogrel in 625 patiënten undergoing non-urgent PCI. Patients were randomized to a clopidogrel-arm (loading dose of 300 mg or 600 mg, followed by 75 mg daily) or an elinogrel-arm (80 mg bolus IV, followed by a bidaily oral maintenance dose of either 50 mg, 100 mg or 150 mg). Using light transmittance aggregometry, it was established that treatment with oral and intravenous elinogrel, resulted in a more rapid antiplatelet effect than clopidogrel in the acute and chronic phases of therapy, without an increase in TIMI-major or minor bleeding. Although the study had insufficient statistical

power to judge clinical endpoints, no difference was observed in combined ischemic endpoints (death, myocardial infarction and stroke) nor in biological endpoints (peri-procedural elevation of myonecrosis markers).

The advantage of both drugs lies in the intravenous administration and the direct onset of action. Although the new orally administered medicaments also result in a rapid onset/offset of platelet inhibition and have been proven effective, we do foresee a role for intravenously administered drugs in patients presenting at the catherization laboratory undergoing primary PCI (STEMI-patients) or patients with established three-vessel-disease to bridge time to surgery. In the latter case the main advance of these intravenous drugs is the rapid reversal of its effect after the end of the infusion and thus the recovery of platelet function. This is subject of the currently enrolling BRIDGE-study (Maintenance of Platelet inihiBition With cangRelor After dIscontinuation of ThienopyriDines in Patients Undergoing surgery; NCT00767507). During the switch from cangrelor to clopidogrel the platelet function is partially restored, and during the switch from intravenous to oral elinogral the magnitude platelet inhibition remains equal. Therefore, it seems that cangrelor is surpassed by elinogrel.

Monitoring of P2Y12-antagonists

There is growing evidence that platelet function tests are capable to identify patients at higher risk of atherothrombotic events. Although multiple platelet function tests are available to capture the efficacy of clopidogrel (**Table 3**), there is currently no consensus regarding the most appropriate method to quantify the magnitude of on-treatment platelet reactivity. Recently, the POPular-study (*The Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI)* , compared multiple platelet function tests in a population of 1069 patients with established coronary artery disease undergoing elective PCI with stent implanting , with the aim to evaluate the ability of multiple platelet function tests in predicting atherothrombotic events.[14] The gold standard light transmittance aggregometry (LTA), which is hampered by its labour intensiveness and the fact that it can only be performed in specialized laboratories, was compared to several point-of-care tests; the VerifyNow® P2Y12, Plateletworks®, PFA-100 COL/ADP, PFA INNOVANCE P2Y*, IMPACT-*R* and IMPACT-*R* ADP. Of the platelet function tests assessed only LTA, VerifyNow®, Plateletworks® and PFA INNOVANCE® P2Y* were significantly associated with the primary endpoint. However, the predictability of these four tests was only modest. None of the tests provided accurate prognostic information to identify patients at higher risk of bleeding.

The negative predictive value (NPV) of the tests was remarkably high and varied between 90 and 94%, whereas the positive predictive value was low (around 12%). The majority of patients responded well to clopidogrel and had a normal on-clopidogrel platelet reactivity (depending on the test up to 75%). Given the high NPV, these patients have a very low risk of atherothrombotic events. Switching to a more potent P2Y12-antagonist would result in a minimum further cardiovascular risk reduction at the cost of a higher bleeding risk. Consequently, in patients with

a normal on-clopidogrel platelet reactivity the continuation of clopidogrel is preferred. In those patients exhibiting high on-clopidogrel platelet reactivity, switching to more potent medication (prasugrel or ticagrelor) might be considered.

Tailoring therapy

Whereas the evidence that high on-treatment platelet reactivity strongly influences the occurrence of atherothrombotic events is overwhelming; data concerning the benefit of tailoring therapy based on the results of platelet function testing are limited.

Only a couple of small studies suggest that individualizing therapy based on platelet function might improve outcome.[40,41] Taking the lack of randomized clinical trials into account, the most recent ESC-guidelines therefore state that platelet function testing remains reserved to research and should not be implemented in daily clinical practice.[42] On the contrary, the American College of Cardiology (ACC), the American Heart Association (AHA), and the Society for Cardiovascular Angiography and Interventions (SCAI) recommend the following (Class IIb, level of evidence C): "In patients in whom stent thrombosis may be catastrophic or lethal (unprotected left main, bifurcating left main, or last patent coronary vessel), platelet aggregation studies may be considered and the dose of clopidogrel increased to 150 mg per day if <50% inhibition of platelet aggregation is demonstrated".[43] It is noteworthy that neither of the guidelines take a position on the platelet function test that should be used (nor on the accompanying cut-off) to identify patients at higher risk.

Nonetheless, during the scientific sessions of the American Heart Association 2010, the GRAVITAS-study (The Gauging Responsiveness With a VerifyNow® Assay-IMPACT on Thrombosis and Safety) was presented as Late Breaking Clinical Trial. GRAVITAS is a prospective, randomized, three-arm, multi-center trial that enrolled 5429 patients undergoing PCI with DES implantation. Patients exhibiting high on-treatment platelet reactivity 12-24 hours post-PCI (defined as PRU>230) [n=2214] were randomized to either standard maintenance therapy (75 mg) or to an additional loading dose of 600 mg and a double maintenance dose (150mg). Outcome measurements included platelet function testing using the VerifyNow, time to major adverse cardiac events (cardiovascular death, nonfatal myocardial infarction or definite/probable ST and non- coronary artery bypass grafting [CABG]- related bleeding) at 30-days and 6-months. The primary endpoint, a composite of cardiovascular death, myocardial infarction and stent thrombosis) was equal in both treatment arm (2.3%). Thus, GRAVITAS demonstrated no benefit of doubling the clopidogrel dose in preventing cardiovascular events in patients with high on-clopidogrel platelet reactivity. Still, these findings do not rule out the benefit of tailor made therapy based on platelet-function testing. Since doubling the dose resulted in only a modest reduction of platelet reactivity and after 30-days even 62% of the patient still suffered from high on-clopidogrel platelet reactivity, a strategy based the use of novel, more potent antiplatelet medication (ie prasugrel or ticagrelor) might be more beneficial. The latter is the subject of investigation in the currently ongoing The Testing Platelet Reactivity

In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel (TRIGGER-PCI) (NCT00910299), aiming to elucidate the efficacy of prasugrel versus clopidogrel in reducing adverse cardiovascular outcomes in patients exhibiting high on-clopidogrel platelet reactivity (defined as PRU>208 at 4 hours post-loading). Approximately 2150 patients with high on-treatment platelet reactivity (defined as PRU>208) undergoing DES-implantation will be enrolled and will be randomized to either prasugrel (60mg loading dose and 10mg maintenance) or to clopidogrel (75 mg maintenance). However, the logical drawback of efficient platelet inhibition is the risk of bleeding complications and it has been suggested that measuring platelet function might be the solution to define a therapeutic window between bleeding and thrombotic events. Taking the costs and risks associated with bleeding into account, we consider platelet function testing a better option as compared to prescribing all patients more potent antiplatelet therapy. In that case, point-of-care platelet function testing is obviously preferred.

Monitoring antiplatelet therapy in patients undergoing percutaneous coronary intervention

This thesis addressed aimed to provide insight into platelet function testing and its clinical applicability. **Part I** of this thesis described the ability of multiple platelet function tests in predicting clinical outcome in patients on dual antiplatelet therapy undergoing elective coronary stent implantation. First, various parameters of classic light transmittance aggregometry (LTA) were compared (chapter 1). Second, platelet function tests assessing the efficacy of clopidogrel (chapter 2 and 3), aspirin (chapter 4) or both (chapter 5) were evaluated.

LTA is considered the 'gold standard' to quantify platelet reactivity, but it remains poorly standardized and various parameters are used by different laboratories to determine the magnitude of on-treatment platelet reactivity. ***Chapter 1*** evaluated the most accurate parameter of LTA. In the first part, the use of native with platelet count-adjusted platelet rich plasma (PRP) was compared, demonstrating that both procedures share equal predictability for adverse clinical outcome. In the second part, it was shown that maximal " ('peak') and late aggregation are able to identify patients at risk with equivalent accuracy, indicating that peak and late aggregation might be interchangeable. Since LTA using native platelet rich plasma is easier to perform and has a similar accuracy in predicting atherothrombotic events, we advise not to adjust the platelet count in platelet rich plasma when predicting thrombotic events after PCI. We leave the choice between peak and late aggregation to the laboratories. However, LTA seems not suitable for routine use in clinical practice because of some major limitations. Therefore, several more easy to use 'point-of-care' platelet function tests have been introduced. Still, the role and clinical applicability of platelet function testing in routine practice remained to be established.

In ***chapter 2***, a head-to-head comparison of multiple platelet-function tests, gauging the antiplatelet efficacy of clopidogrel, was described. In the POPular-study (*Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI)* 'gold standard' LTA was compared with the VerifyNow® P2Y12, Plateletworks, IMPACT-R, IMPACT -R ADP, PFA-100

COL/ADP and INNOVANCE PFA® P2Y. It was demonstrated that only LTA, VerifyNow®, Plateletworks® and INNOVANCE® PFA P2Y* were able to predict the likelihood of an atherothrombotic event in patients undergoing elective PCI with stent implantation. Of importance, the predictability of these four tests was only modest and therefore the POPular-study did not support the use of platelet function testing to guide clinical practice in a low-risk population of patients undergoing elective PCI. Another observation was that none of the tests provided accurate prognostic information to identify patients at higher risk of bleeding. In **chapter 3** a meta-analysis of six studies including the POPular is described. In this systematical evaluation of the significance of on-clopidogrel platelet reactivity in predicting clinical outcome using the VerifyNow®-system, it was confirmed that the magnitude of on-clopidogrel platelet reactivity measured by the VerifyNow®-system is associated with long-term clinical outcome.

Aspirin is the most widely used drug and plays a major role in the prevention of cardiovascular, cerebrovascular , and peripheral vascular related events. In ***chapter 4*** platelet function tests assessing the efficacy of aspirin were described. It was clearly demonstrated that AA-induced LTA and the VerifyNow® ASA test, both aggregation-based tests sensitive for COX-1 activity using arachidonic acid as the agonist, were able to predict clinical outcome. Of these test, the VerifyNow had the highest predictive accuracy. Like the platelet function tests specific for clopidogrel, none of these tests were able to identify patients at higher risk of bleeding. In ***chapter 5*** the incidence of high on-treatment platelet reactivity to both aspirin and clopidogrel simultaneously and its association with clinical outcome was explored. The tests that were capable to predict clinical outcome with both the clopidogrel and the aspirin version were used. It was determined that dual high on-treatment platelet reactivity was more prevalent than previously assumed. In addition, patients with dual high on-treatment platelet reactivity carried the highest risk of adverse events.

In **part II** the effect of several patient characteristics on the magnitude of platelet reactivity and clinical outcome was described. ***Chapter 6*** described the influence of high on-treatment platelet reactivity on clinical outcome in patients with diabetes mellitus. In the present study including diabetic patients with established coronary artery disease undergoing elective PCI, assessment of platelet reactivity was not associated with significant rates of death, myocardial infarction, stent thrombosis or stroke. Thus, the use of high on-treatment platelet reactivity did not improve classification of individuals into clinically relevant risk categories. This suggests that in patients suffering from diabetes mellitus undergoing *elective* PCI, assessment of platelet reactivity does not improve prediction of clinical outcome. In ***chapter 7*** the impact of renal function on the magnitude of platelet reactivity and subsequent clinical outcome in patients undergoing percutaneous coronary intervention with stenting was described. The present analysis demonstrated that both the magnitude of platelet reactivity as well as the incidence of high on-clopidogrel platelet reactivity was higher in patients with chronic kidney disease. Of importance, patients with both an impaired renal function and high on-clopidogrel platelet reactivity carried the highest risk of long-term cardiovascular events, suggesting the need for tailoring therapy in these high-risk patients.

In ***chapter 8*** the effect of gender on platelet reactivity and the incidence of high on-treatment platelet reactivity was investigated. Throughout the last decade improvements in the diagnosis and treatment of atherosclerosis have caused a marked reduction in the morbidity and mortality in men, whereas the rate of recurrent atherothrombotic events, including cardiovascular death, in women has increased. Previous studies have suggested that women do not accrue equal therapeutic benefit of antithrombotic therapy. However, the physiological mechanism behind this gender disparity remains unclear. Therefore, the aim of the present study was to compare the magnitude of on-treatment platelet reactivity between genders in patients on dual antiplatelet therapy undergoing elective coronary stenting. Although we support the finding that the magnitude of platelet reactivity is higher in women, the absolute difference between genders is small and both the cut-off value to identify patients at risk and the incidence of the composite endpoint was similar between genders. Thus, it is unlikely that the difference in platelet reactivity accounts for a worse prognosis in women. In ***chapter 9*** a population presenting with a ST-elevation myocardial infarction (STEMI) was presented. Although it is well known that both heightened platelet reactivity and an occluded infarct-related artery on initial angiography and at the time of primary PCI are associated with a worsened clinical outcome in patients presenting with STEMI, the relation between platelet reactivity and the infarct-related artery patency has not been established yet. Data from this analysis suggested that activated platelets and leucocytes play a role in the pathophysiological process leading to infarct related artery occlusion. Because of the superior clinical outcomes associated with an open infarct related artery at initial angiography, combined with the knowledge that additional antithrombotic therapy can achieve coronary reperfusion, these results should encourage the use of more potent antithrombotic therapy early after the onset of symptoms into obtain early recanalization of the IRA. In ***chapter 10*** it iwas investigated whether patients with a history of stent thrombosis (ST) exhibit a heightened platelet reactivity phenotype. Since stent thrombosis is associated with a high recurrence rate and considerable mortality, it remains the dark site of coronary stenting . The pathophysiology of ST has evolved from the identification of single causative factors to a complex multifactorial origin. Predictors can be categorized as clinical, procedural, or lesion related. Recently, the involvement of novel determinants has been recognized, including an impaired responsiveness to antiplatelet therapy and a heightened platelet reactivity status despite antiplatelet therapy. In the present analysis, it was established that patients with a history of early stent thrombosis exhibit a poor responsiveness to clopidogrel and a heightened on-clopidogrel platelet reactivity. In addition, both early as well as late stent thrombosis were strongly and independently associated with a heightened on-aspirin platelet reactivity and dual antiplatelet therapy resistance was frequent in this particular subset of patients. ***Chapter 11*** described three subgroups of patients having a higher risk of bleeding during treatment with the thienopyridine prasugrel were investigated; patients with a history of stroke or transient ischemic attack (TIA), patients ≥75 years, and patients with a body weight < 60 kg. The higher bleeding rate may be due to a stronger prasugrel induced inhibition of platelet aggregation in these subgroups. However,

prasugrel has been introduced only recently and thus, little pharmacodynamic data are available. The aim of the present analysis was to investigate the influence of another thienopyridine; clopidogrel. It was established that in two high-risk subgroups for bleeding, patients ≥75 years and patients with previous stroke, on-clopidogrel platelet reactivity was increased. In contrast, in patients with a low body weight, on-clopidogrel platelet reactivity was decreased, suggesting that only in patients with low body weight a stronger response to a thienopyridine might lead to more bleeds.

IN CONCLUSION

Coronary artery disease is the main cause of mortality and morbidity worldwide. Throughout the last decade improvements in the prevention, diagnosis and treatment of acute coronary syndromes (ACS) have caused a marked reduction in the rate of recurrent atherothrombotic events, including cardiovascular death. Given the pivotal role of platelets in thrombus formation and atherosclerosis, dual antiplatelet therapy with both aspirin and clopidogrel has become the cornerstone in the treatment of patients undergoing coronary stent implantation and those presenting with ACS. The individual response to clopidogrel is highly variable and multiple studies have been linking a high on-treatment platelet reactivity to atherothrombotic events. It has been hypothesized that individual monitoring of platelet reactivity and decreasing the magnitude of platelet reactivity below this threshold might improve clinical outcome.[14] Novel P2Y12-antagonists have the advantage of a more rapid onset, and a less variable, more consistent platelet inhibition.[32,36] To reduce peri-procedural complications and improve clinical outcome, these drugs will be introduced in both the guidelines and daily clinical practice. Since the logical drawback of more potent platelet inhibition is an increased bleeding risk, these novel medication seems not indicated for everyone and thus we have introduced the POPular risk score. This score establishes whether patients are adequately treated while on clopidogrel or need more potent antiplatelet therapy (the recently introduced prasugrel). Since atherosclerosis and atherothrombosis is a multifactorial phenomenon, this score comprises the magnitude of platelet reactivity, the periprocedural risk, the vascular state as well as a couple of classic risk factors. In addition, polymorphism of the genes encoding the metabolism of clopidogrel are included. (**Figure 2**). The POPular-risk score can establish which medication is more appropriate, balancing between the risk of atherothrombotic and bleeding events, navigating between Scylla and Charybdis.

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Samenvatting

SAMENVATTING

Bloedplaatjes spelen een cruciale rol bij de stolling van bloed en de reparatie van de bloedvaatwand na een beschadiging. Daarom worden patiënten die een percutane coronaire interventie (PCI) met stent-implantatie ondergaan of zich presenteren met een acuut coronair syndroom (ACS), standaard behandeld met de plaatjesaggregatie-remmers aspirine en clopidogrel. Clopidogrel wordt echter geplaagd door een aanzienlijke interindividuele variabiliteit in respons en een gedeelte van de patiënten reageert onvoldoende op dit medicijn. Een hoge mate van bloedplaatjesreactiviteit ondanks medicamenteuze behandeling is geassocieerd met het optreden van het ontstaan van trombose in atherosclerotische slagaders (onder andere van het hart) met daardoor complicaties in de vorm van een hartinfarct, een beroerte of sterfte. Het is ter voorkoming van complicaties aldus van belang te weten of de patiënt goed reageert op clopidogrel en er is dan ook veel aandacht voor het monitoren van de individuele mate van de plaatjesreactiviteit met zogenaamde bloedplaatjesfunctietesten. Er zijn meerdere testen op de markt om de bloedplaatjesfunctie te meten. Echter, tot op heden is niet goed bekend of deze testen in de dagelijkse praktijk uitvoerbaar zijn, of ze inderdaad complicaties na een dotterbehandeling voorspellen en indien dit zo is, welke test hierin het beste is.

Deel I van dit proefschrift beschrijft het vermogen van verschillende bloedplaatjesfunctietesten om klinische eindpunten te voorspellen in een populatie patiënten die een geplande coronaire stent-plaatsing ondergaan. Eerst worden verschillende parameters van de klassieke licht transmissie aggregatie (LTA) met elkaar vergeleken (hoofdstuk 1). Vervolgens worden plaatjesfunctietesten die gevoelig zijn voor het effect van clopidogrel (hoofdstuk 2 en 3), aspirine (hoofdstuk 4) of beide (hoofdstuk 5) geëvalueerd.

Licht transmissie aggregatie (LTA) wordt gezien als de 'gouden standaard', maar is naast arbeidsintensief ook slecht gestandaardiseerd. Diverse parameters worden door verscheidene laboratoria gebruikt om de mate van plaatjesreactiviteit vast te stellen. ***Hoofdstuk 1*** gaat in op de mate waarin LTA klinische eindpunten kan voorspellen, bij gebruik van deze verschillende parameters. In het eerste deel, wordt het gebruik van plasma dat is gestandaardiseerd (verdund) naar een aantal van 250.000 bloedplaatjes/µL vergeleken met gebruik van onbehandeld plasma. Beide procedures delen een vergelijkbare mate van voorspelbaarheid. In het tweede gedeelte, wordt de maximale amplitude van de aggregatiecurve (piek aggregatie) vergeleken met de absolute mate van late aggregatie. Ook deze twee meetpunten zijn even voorspellend. Deze bevindingen suggereren dat het weinig uitmaakt welke van de parameters gekozen wordt bij de bepaling of clopidogrel een voldoende mate van bloedplaatjesremming teweeg heeft gebracht. Aangezien het proces van standaardiseren van plasma tijdrovend is, adviseren wij gebruik te maken van niet-verdund plasma. De keuze voor piek of late aggregatie laten we over aan het betreffende laboratorium.

Hoofdstuk 2 beschrijft de POPular (*Do **P**latelet Function Assays Predict Clinical **O**utcomes in clopidogrel Pretreated patients undergoing elective **P**CI)*. De POPular heeft, in een populatie van 1069 patiënten die een geplande PCI ondergingen, verscheidene testen naast elkaar gelegd. De traditionele test, licht transmissie aggregatie, die voorbehouden is aan gespecialiseerde laboratoria en arbeidsintensief is, werd vergeleken met een aantal tests die direct aan het bed van de patiënt gebruikt kunnen worden; de VerifyNow® P2Y12; IMPACT-*R*, IMPACT -*R* ADP; PFA-100 COL/ADP; Plateletworks®; INNOVANCE PFA® P2Y. Beoordeeld werd of de testen in staat waren om de complicaties sterfte, hartinfarct, ischemisch CVA en stent trombose te voorspellen. Ook werd onderzocht of er patiënten waren die overmatig reageerden op clopidogrel en daardoor een bloeding kregen. Van de onderzochte testen bleken licht transmissie aggregatie, de VerifyNow® P2Y12-assay, Plateletworks® en de INNOVANCE PFA® P2Y in staat om het optreden van complicaties te voorspellen. De overige testen hadden geen voorspellende waarde. Geen van de testen was geschikt om onderscheid te maken tussen patiënten bij wie wel of geen bloeding optrad. De conclusie van de POPular-studie is dat we nu vier testen in handen hebben – waarvan er twee eenvoudig aan het bed van de patiënt gebruikt kunnen worden – die complicaties na een dotterbehandeling voorspellen. Alhoewel de voorspelbaarheid van de testen gering was, kan deze uitkomst grote implicaties hebben voor patiënten die een dotterbehandeling moeten ondergaan. Alvorens deze testen bij alle patiënten ingezet kunnen worden, zullen we echter eerst moeten bewijzen dat het zinvol is andere (sterkere) bloedplaatjes remmende medicijnen in te zetten bij patiënten die een onvoldoende response op de huidige bloedplaatjesremmende medicijnen hebben. In ***hoofdstuk 3*** wordt een meta-analyse van een zestal studies, waaronder de POPular, beschreven. Deze systematische analyse bestudeert het belang van de mate van plaatjesreactiviteit zoals gemeten met de VerifyNow® P2Y12-assay bij het voorspellen van klinische uitkomsten. Het bevestigt dat de grootte van plaatjesreactiviteit, ondanks behandeling met clopidogrel, samenhangt met langetermijnscomplicaties.
Aspirine is wereldwijd het meest gebruikte medicijn en speelt een cruciale rol in de preventie van vaatgerelateerde complicaties. In ***hoofdstuk 4*** worden vier bloedplaatjesfunctietesten beschreven die de effectiviteit van aspirine bepalen. Klassieke LTA en de VerifyNow®Aspirine zijn beide in staat om uitkomsten te voorspellen. Van deze twee testen had de VerifyNow®Aspirine de hoogste voorspellende waarde. Net als de bloedplaatjesfunctietesten specifiek voor het effect van clopidogrel, zijn ook deze testen niet in staat om het optreden van bloedingen te voorspellen.

In ***hoofdstuk 5*** wordt gebruik gemaakt van de testen die zowel met de versie specifiek voor clopidogrel als de testen specifiek voor aspirine klinische eindpunten kunnen voorspellen. Allereerst wordt de incidentie van het gelijktijdig optreden van onvoldoende bloedplaatjesfunctie-remming door clopidogrel en aspirine bestudeerd. Vervolgens wordt bepaald of deze 'dubbele' onvoldoende remming is geassocieerd met uitkomsten. Het blijkt dat het gelijktijdig optreden van onvoldoende remming door beide medicijnen niet alleen vaker voorkomt dan voorheen werd aangenomen, maar dat het bovendien samengaat met het hoogste risico op trombotische complicaties.

In **deel II** wordt de invloed van verschillende patiëntenkarakteristieken op de mate van plaatjesreactiviteit beschreven. In ***hoofdstuk 6*** wordt de subpopulatie patiënten met diabetes mellitus bestudeerd. In deze studie met diabetes patiënten met vastgesteld kransslagaderlijden die een geplande PCI ondergingen, was de plaatjesreactiviteit niet geassocieerd met dood, myocard infract, stent trombose of CVA. Dit suggereert dat in diabetici een hoge mate van plaatjesreactiviteit niet bijdraagt aan het voorspellen van klinische uitkomsten na revascularisatie. De verklaring hiervoor ligt mogelijk in de kwaliteit van het hartteam dat in staat blijkt om de juiste kandidaten voor revascularisatie middels PCI of CABG te selecteren.

Hoofdstuk 7 onderzoekt het effect van nierfunctie op de grootte van de plaatjesreactiviteit en de daarmee samenhangende klinische uitkomsten. De plaatjesreactiviteit is hoger in patiënten met matig/ernstig nierfalen. Ook hebben deze patiënten vaker onvoldoende plaatjesaggregatieremming door clopidogrel. De voornaamste bevinding is dat patiënten die naast nierfalen ook een onvoldoende remming van de bloedplaatjesfunctie hebben, het hoogste risico dragen op het krijgen van complicaties. Dit suggereert dat patiënten met nierfalen wellicht gebaat zijn bij een sterkere plaatjesremmende therapie.
In de laatste decennia hebben verbeteringen in de diagnose en behandeling van atherosclerose (slagaderverkalking) geleid tot een forse afname in de morbiditeit en mortaliteit van mannen, terwijl het aantal trombotische complicaties in vrouwen onverminderd hoog blijft. Verscheidene studies hebben de suggestie gewekt dat vrouwen wellicht minder baat hebben bij antitrombotische therapie in de vorm van aspirine en clopidogrel. Het is echter onduidelijk waardoor dit verschil in effectiviteit veroorzaakt wordt. Daarom wordt in ***hoofdstuk 8*** de invloed van gender op de mate van plaatjesreactiviteit en het voorkomen van een onvoldoende remming door clopidogrel onderzocht. Hoewel we bevestigen dat vrouwen een hogere bloedplaatjesreactiviteit hebben, is het absolute verschil klein en is ook het optreden van complicaties gelijk tussen mannen en vrouwen. Hierdoor lijkt het onwaarschijnlijk dat het verschil in plaatjesreactiviteit de verklaring vormt voor de minder goede prognose van vrouwen na een PCI.

In ***hoofdstuk 9*** wordt een populatie patiënten met een ST-elevatie myocard infarct (STEMI) gepresenteerd. Hoewel reeds is beschreven dat zowel een hoge mate van plaatjesreactiviteit en een geocludeerde infarct-gerelateerde arterie ten tijde van een primaire PCI samenhangen met een slechtere prognose, is de relatie tussen plaatjesreactiviteit en mate van vernauwing van de infarct-gerelateerde arterie nog onbekend. Data uit deze analyse tonen aan dat geactiveerde bloedplaatjes en leukocyten een rol spelen in het pathofysiologische proces leidend tot een occlusie. Gezien de betere uitkomsten bij een open infarct-gerelateerde coronair bij angiografie, gecombineerd met de wetenschap dat extra antitrombotische therapie vlotte reperfusie kan bewerkstelligen, zouden deze bevindingen het gebruik van meer potente antitrombotische therapie vroeg na het optreden van symptomen moeten aanmoedigen.

In ***chapter 10*** wordt onderzocht of patienten die een stent trombose hebben doorgemaakt een verhoogd plaatjesreactiviteit fenotype hebben. Aangezien stent trombose geassocieerd is met een hoge recidiefkans en een aanzienlijke mortaliteit, blijft het de donkere zijde van PCI met stent implantatie. De pathofysiologie heeft zich inmiddels ontwikkeld van de identificatie van enkele causatieve factoren tot een complex multifactorieel model. Voorspellers van stent trombose kunnen worden ingedeeld klinisch, procedureel of lesie-gerelateerd. Recentelijk zijn nieuwe determinanten vastgesteld, waaronder een verminderde respons op antiplaatjestherapie en een hoge mate van plaatjesreactiviteit ondanks medicatie. In de huidige analyse wordt aangetoond dat patienten met een voorgeschiedenis van vroege stent trombose een verminderde reactie op clopidogrel hebben en een onvoldoende bloedplaatjesfunctie-remming door aspirine. Ook wordt duidelijk dat zowel vroege als late stent trombose onafhankelijke voorspellers zijn van onvoldoende bloedplaatjesfunctie-remming door aspirine en dat het gelijktijdig optreden van onvoldoende bloedplaatjesfunctie-remming door clopidogrel en aspirine frequent voorkomt in deze speciale categorie patiënten.

Hoofdstuk 11 beschrijft een drietal groepen waarvan is vastgesteld dat zij een hoger bloedingsrisico hebben wanneer zij behandeld wordt met de nieuwe thienopyridine prasugrel. Dit is patiënten die een cerebrovasculair event (CVA) of TIA hebben doorgemaakt, patiënten ouder dan 75 jaar en patiënten met een lichaamsgewicht onder de 60 kg. Het hogere bloedingsrisico in deze specifieke groepen zou toegeschreven kunnen worden aan een sterkere prasugrel-geïnduceerde plaatjesremming. Aangezien prasugrel recent is geïntroduceerd, is er slechts weinig bekend over de farmacokinetiek. In het huidige onderzoek, met het doel het effect van de andere thienoypyridine clopidogrel te bestuderen, werd vastgesteld dat in twee groepen met een verhoogd bloedingsrisico, namelijk patiënten ouder dan 75 jaar en patiënten met een doorgemaakt CVA/TIA, de plaatjesreactiviteit verhoogd is. In patiënten met een laag lichaamsgewicht daarentegen, is de bloedplaatjesreactiviteit verlaagd. Deze bevindingen suggereren dat enkel in patiënten met een laag lichaamsgewicht een sterkere reactie op een thienopyridine tot meer bloedingen kan leiden.

In ***hoofdstuk 12*** wordt afgerond met een bespreking van de klinische toepasbaarheid van plaatjesfunctiesten en wordt vooruitgeblikt naar de nieuwe mogelijkheden op het gebied van plaatjesaggregatieremmende medicatie.

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Publications

Publications

- **Breet NJ**, Donkersgoed HE, van Werkum JW, Bouman HJ, Kelder JC, Zijlstra F, Hackeng CM, ten Berg JM. Is platelet inhibition due to thienopyridines increased in elderly patients, in patients with previous stroke and patients with low body weight as a possible explanation of an increased bleeding risk? Accepted for publication in Neth Heart J.
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Curriculum Vitae

CURRICULUM VITAE

Nicolien Breet werd 14 juni 1980 geboren in Harmelen en verhuisde op jonge leeftijd naar het noorden van het land, waar zij genoot van een onbezorgde jeugd met ruimte voor muziek (piano), sport en spel.

Na met veel plezier het gymnasium in Assen te hebben doorlopen (CSG Vincent van Gogh), werd zij in 1998 door de numerus fixus gedwongen uit te wijken naar een studie psychologie aan de Rijksuniversiteit Groningen. Na het afronden van psychologie (majors Sociale en Organisatie Psychologie en minor Klinische Psychologie), startte zij in 2002 met de studie geneeskunde. In mei 2006 behaalde zij haar doctoraal examen (*cum laude*) en in december 2007 werd zij arts (*cum laude*). Aansluitend was zij werkzaam als arts-assistent cardiologie in het St.Antonius Ziekenhuis te Nieuwegein, waar zij datzelfde jaar aan haar opleiding tot cardioloog begon (opleider dr. W. Jaarsma/ dr. J.M. ten Berg). In 2009 begon zij aan het onderzoek dat resulteerde in dit proefschrift. Na haar promotie zal zij haar opleiding voortzetten bij de vooropleiding interne geneeskunde, eveneens in het St.Antonius Ziekenhuis (opleider: dr.A.B.M.Geers) en hoopt zij cardioloog te worden in 2016.

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