

## ORIGINAL ARTICLE

# Phenotyping vs. genotyping for prediction of clopidogrel efficacy and safety: the PEGASUS-PCI study

J. M. SILLER-MATULA,\* G. DELLE-KARTH,\* I. M. LANG,\* T. NEUNTEUFL,\* M. KOZINSKI,† J. KUBICA,† G. MAURER,\* K. LINKOWSKA,‡ T. GRZYBOWSKI,‡ K. HUBER§ and B. JILMA¶

\*Department of Cardiology, Medical University of Vienna, Vienna, Austria; Departments of †Cardiology and Internal Medicine and ‡Institute of Molecular and Forensic Genetics, Collegium Medicum of the Nicolaus Copernicus University, Bydgoszcz, Poland; §3rd Medical Department of Cardiology and Emergency Medicine, Wilhelminen Hospital, Vienna; and ¶Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria

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**Summary.** *Background:* Prognostic values of genotyping and phenotyping for assessment of clopidogrel responsiveness have been shown in independent studies. *Objectives:* To compare different assays for prediction of events during long-term follow-up. *Methods:* In this prospective cohort study polymorphisms of CYP2C19\*2 and CYP2C19\*17 alleles, vasodilator-stimulated phosphoprotein phosphorylation (VASP) assay, multiple electrode aggregometry (MEA), cone and platelet analyser (CPA) and platelet function analyser (PFA-100) were performed in 416 patients undergoing percutaneous coronary intervention. The rates of events were recorded during a 12-month follow-up. *Results:* Platelet aggregation by MEA predicted stent thrombosis (2.4%) better (c-index = 0.90;  $P < 0.001$ ; sensitivity = 90%; specificity = 83%) than the VASP assay, CPA or PFA-100 (c-index < 0.70;  $P > 0.05$ ; sensitivity < 70%; specificity < 70% for all) or even the CYP2C19\*2 polymorphism (c-index < 0.56;  $P > 0.05$ ; sensitivity = 30%; specificity = 71%). Survival analysis indicated that patients classified as poor responders by MEA had a substantially higher risk of developing stent thrombosis or MACE than clopidogrel responders (12.5% vs. 0.3%,  $P < 0.001$ , and 18.5% vs. 11.3%,  $P = 0.022$ , respectively),

whereas poor metabolizers (CYP2C19\*1/\*2 or \*2/\*2 carriers) were not at increased risks (stent thrombosis, 2.7% vs. 2.5%,  $P > 0.05$ ; MACE, 13.5% vs. 12.1%,  $P = 0.556$ ). The incidence of major bleedings (2.6%) was numerically higher in patients with an enhanced vs. poor response to clopidogrel assessed by MEA (4% vs. 0%) or in ultra-metabolizers vs. regular metabolizers (CYP2C19\*17/\*17 vs. CYP2C19\*1/\*1; 9.5% vs. 2%). The classification tree analysis demonstrated that acute coronary syndrome at hospitalization and diabetes mellitus were the best discriminators for clopidogrel responder status. *Conclusions:* Phenotyping of platelet response to clopidogrel was a better predictor of stent thrombosis than genotyping.

**Keywords:** clopidogrel, CYP2C19, major bleeding, MEA, platelets, polymorphism, sensitivity, specificity, stent thrombosis.

Correspondence: Bernd Jilma, Department of Clinical Pharmacology, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

Tel.: +43 1 404002981; fax: +43 1 404002998.

E-mail: bernd.jilma@meduniwien.ac.at

Correspondence: Jolanta Siller-Matula, Department of Cardiology, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

Tel.: +43 1 404004614; fax: +43 1 404004216.

E-mail: jolanta.siller-matula@meduniwien.ac.at

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## Introduction

Clopidogrel is an irreversible platelet inhibitor representing a mainstay treatment for patients undergoing coronary stenting [1]. Although clopidogrel is effective in the secondary prevention of atherothrombotic events, its limitations, including high inter-individual variability of response [2–5] and potential for drug-drug interactions [6–8], led to the development of novel platelet inhibitors [9]. Nevertheless, clopidogrel currently remains the ‘gold standard’ antiplatelet agent in patients undergoing elective percutaneous coronary intervention (PCI). Insufficient platelet inhibition by clopidogrel, which is an independent predictor of ischemic events, can be detected by a number of laboratory methods. As clopidogrel is metabolized by a highly polymorphic cytochrome P450 (CYP) system in the liver, genotyping, especially of the CYP2C19 isoenzyme, has been proposed as a possible strategy for identifying patients who might not properly benefit from clopidogrel therapy [10–14]. On the other hand, several methods for phenotyping of the pharmacodynamic

effect of clopidogrel might represent alternative diagnostic options [15,16]. A general problem with assessing platelet inhibition by clopidogrel is, however, that no single assay encompasses the complexity of platelet physiology. Due to the lack of data regarding which assay might best predict adverse events, prospective comparisons are needed. Indeed, several studies compared different tests for assessment of the clopidogrel effect in terms of between-assay agreement and correlation [15,17–21]. Although direct comparisons between methods for prediction of adverse events during clinical follow-up are of most interest, studies providing such data are scarce [10,16,22]. Recently, predictive values for tests assessing the phenotype of clopidogrel effect [16,23] and of the genotyping of the CYP2C19 allele have been shown in independent studies [24,25]. However, a direct comparison between both approaches is to our knowledge missing. Therefore, we compared the diagnostic accuracy of phenotyping vs. genotyping for prediction of ischemic and bleeding events in patients with coronary artery disease undergoing percutaneous coronary intervention during 1 year follow-up.

## Methods

### Study design

The PEGASUS-PCI study (PhEnotyping versus Genotyping for prediction of cardiac AdverSe events in patients Undergoing Percutaneous Coronary Intervention) was a prospective observational cohort study performed at the Medical University of Vienna. The Ethics Committee of the Medical University of Vienna approved the study protocol in accordance with the Declaration of Helsinki. Participants were included in the study between March 2007 and September 2008, and followed-up until November 2009. Clinical follow-up information was obtained by contacting all patients by phone and/or mail at 3, 6, 9 and 12 months. Source documents of potential events were obtained. Additionally, information concerning the cause of death was obtained from the national death registry (Statistics Austria). Data have been collected until September 2010. Inclusion criteria were: written informed consent obtained before the study entry, stent implantation, PCI at least 2 h after clopidogrel loading with 600 mg, age > 18 years and planned treatment with clopidogrel and aspirin for 12 months. The only exclusion criterion was participation in interventional trials. The study population was a consecutive series of participants defined by the selection criteria. Four hundred and sixteen patients with coronary artery disease (CAD) undergoing PCI were consecutively enrolled. All patients received a clopidogrel loading dose of 600 mg followed by a daily dose of 75 mg. The vast majority of patients (99%) received a drug-eluting stent. All interventions were performed according to current standard guidelines, and the type of stent implanted was at the discretion of the interventional cardiologist. Blood samples from patients were obtained from the arterial sheath (6F) in the catheterization laboratory directly post-PCI and at least 5 min after intravenous infusion of aspirin. Functional platelet assays were

performed directly after blood sampling whereas the VASP assay was performed up to 24 h after blood sampling at the Department of Clinical Pharmacology at the Medical University of Vienna. Patients receiving GPIIb/IIIa inhibitors ( $n = 14$ ) have been excluded from all analyses regarding functional platelet testing. Genotyping was performed after inclusion of the last participant at the Institute of Molecular and Forensic Genetics, Collegium Medicum of the Nicolaus Copernicus University in Bydgoszcz, Poland. All analyses were performed by trained laboratory technicians blinded to the results of other tests and to the outcomes. All tests were performed in each participant.

The study is reported according to the STARD (standards for the reporting of diagnostic accuracy studies) and STROBE (strengthening the reporting of observational studies in epidemiology) standards.

### Analysis of VASP phosphorylation by flow cytometry

To determine the VASP (vasodilator-stimulated phosphoprotein) phosphorylation state of whole blood, we used a standardized flow cytometric assay (Platelet VASP; BioCytex, Marseille, France). Blood samples collected in 3.8% sodium citrate were incubated *in vitro* with ADP and/or prostaglandin E1 (PGE1) before fixation, according to the manufacturer's instructions. After 10 min, platelets were permeabilized and labeled with a primary monoclonal antibody against serine 239-phosphorylated VASP (clone 16C2) or its isotype, followed by a secondary fluorescein isothiocyanate (FITC)-conjugated polyclonal goat-anti-mouse antibody. All procedures were performed at room temperature. Geometric mean fluorescence intensity (GMFI) was determined using a flow cytometer (FACSCalibur System; BD Biosciences, Vienna, Austria) [26]. The platelet population was identified by its forward and side-scatter distribution, and 10 000 platelet events were gated and analyzed for GMFI. Platelet reactivity was expressed as platelet reactivity index (PRI) calculated as  $PRI\% = [(GMFI(PGE1) - GMFI(PGE1 + ADP))/GMFI(PGE1)] \times 100$ . The ratio is expressed as mean percentage platelet reactivity, inversely correlated with the clopidogrel treatment efficiency. The normal value of the PRI without treatment with ADP antagonists is 69–100% [6]. The VASP assay has been shown to have a high reproducibility, even after repeated testing of the same sample over 24 h [27]. This was reproducible in our study: coefficient of variation for duplicate analysis was 5%.

### Impedance aggregometry

Whole blood aggregation was determined using multiple electrode aggregometry (MEA) on a new generation impedance aggregometer (Multiplate Analyzer; Verum Diagnostica GmbH, Munich, Germany). The system detects the electrical impedance change due to the adhesion and aggregation of platelets on two independent electrode-set surfaces in the test cuvette. We used hirudin as anticoagulant, which is recommended by the manufacturer. We used adenosine diphosphate

(ADP) + prostaglandin E1 (PGE1) or ADP alone as agonists. A 1:2 dilution of whole blood anticoagulated with hirudin and 0.9% NaCl was stirred at 37 °C for 3 min in the test cuvettes, ADP (6.4 µM) and PGE1 (9.4 nM) were added and the increase in electrical impedance was recorded continuously for 6 min. The mean values of the two independent determinations are expressed as the area under the curve of the aggregation tracing (AUC). The MEA instrument allows two ways to express the AUC: as AU\*min (arbitrary aggregation units) or as U (units); 10 AU\*min corresponds to 1 U. The recommendation to express the AUC as U was introduced by the manufacturer in order to simplify the expression of results by providing a more simple unit (U instead of AU\*min) and also by providing smaller numbers. Admittedly, this is causing some confusion in the literature. We reported AUC in units (U) [16]. A good reproducibility of MEA has been reported (< 6% variability) [28]. Patients with values up to 20 U have been classified as ultra-responders, those with 21–47 U as regular responders and those with values > 48 as poor responders. Due to the best predictive values for the ADP + PGE1-induced platelet aggregation by MEA based on receiver operating characteristic (ROC) curve analysis, this test was chosen as a reference for comparisons of diagnostic accuracy.

#### Platelet function analyzer (PFA-100)

The PFA-100 (Dade Behring, Marburg, Germany) was used for measuring platelet function under high shear rates (5000–6000 s<sup>-1</sup>) [29]. Blood samples collected in 3.8% sodium citrate were used. The PFA-100 measures the time required for occlusion of the aperture by platelet plugs, which is defined as closure time (CT). The instrument aspirates a blood sample under constant vacuum from the sample reservoir through a capillary and a microscopic aperture (147 µm) cut into the membrane, which leads to high shear induced platelet plug formation [29]. The membrane is coated with collagen/adenosine diphosphate (CADP). Published data have shown a satisfactory reproducibility of the test. Less than 2% of samples have shown a variation of more than 20% between the repeated measurements [29]. The reference value for CADP-CT in individuals not treated with ADP antagonists is 65–120 s [29].

#### Cone and platelet analyzer (CPA, ImpactR)

The cone and platelet analyzer (DiaMed, Cressier, Switzerland) tests whole blood platelet adhesion and aggregation under flow conditions; 130 µL of whole blood (3.2% citrate) is pre-incubated with agonist (adenosine diphosphate [ADP] 2 µM) during constant mixing for 2 min. Subsequently, blood is placed in a polystyrene well ('plate') and a shear rate of 1800 s<sup>-1</sup> is applied [30]. There are two contact surfaces for blood: 'plate' and 'cone'. The adherent platelets on the 'plate' surface are stained, the percentage of surface coverage (SC) and the average size (AS) of the objects are determined by an image analyzer. Without ADP-antagonists the normal value of SC is

< 4.6% and of AS of platelet aggregates is > 43 µm<sup>2</sup> (J. Siller-Matula and B. Jilma, unpublished data).

#### CYP2C19 genotyping

Genomic DNA was extracted from blood according to the standard procedures. CYP2C19\*17 (CYP2C19\_-806\_C>T, rs12248560) was genotyped with a commercially available validated drug metabolism genotyping assay (TaqMan Drug Metabolism Genotyping Assay C\_469857\_10; Applied Biosystems, Foster City, CA, USA) with the ABI Prism Sequence Detector 7000 (Applied Biosystems) in accordance with manufacturer's instructions. CYP2C19\*2 (CYP2C19\_681\_G>A; rs4244285) was genotyped with real-time allelic discrimination assay on an ABI Prism Sequence Detector 7000 (Applied Biosystems) according to standard procedures. Primers 5'-GATATGCAATAATTTTCCCACTATCATTG-3' and 5'-GGTGTCTTTTACTTTCTCCAAAATATCAC-3' were used to amplify a sequence of the CYP2C19 gene containing the single nucleotide polymorphism 681G>A (rs4244285). The sequence of the G allele-specific probe was 5'-FAM-TTATTTCCCGGGAACC-3' and the sequence of the A allele-specific probe was 5'-VIC-ATTATTTCCCAGGAACC-3'. After PCR, fluorescence yield for the two different dyes was measured and presented in a two-dimensional graph to obtain the allelic discrimination plot and identify individual genotypes. Correctness of genotyping was evaluated for randomly selected samples by direct sequencing of PCR products with the use of the BigDye Terminator v. 3.1 sequencing kit and 3130xl Genetic Analyzer (Applied Biosystems). No discrepancies were observed between real-time discrimination and sequencing strategies. Patients with a loss of function CYP2C19\*2 allele were classified as poor metabolizers (CYP2C19\*1/\*2, heterozygote poor metabolizers; CYP2C19\*2/\*2, homozygote poor metabolizers), whereas patients with a gain of function CYP2C19\*17 allele were classified as ultra-metabolizers (CYP2C19\*1/\*17, heterozygote ultra-metabolizers; CYP2C19\*17/\*17, homozygote ultra-metabolizers) [31]. Patients with a CYP2C19\*1 allele were classified as regular metabolizers and diplotypes with CYP2C19\*2/\*17 allele were classified as mixed metabolizers.

#### Study endpoints

The primary efficacy endpoint was the incidence of stent thrombosis (definite and probable) during a 12-month follow-up. Definite stent thrombosis was defined according to the Academic Research Consortium criteria as the occurrence of an acute coronary syndrome with either angiographic or pathological confirmation of thrombosis [32]. Probable stent thrombosis was defined as any unexplained death within 30 days or target vessel MI without angiographic confirmation of thrombosis or other identified culprit lesion [32]. The primary safety endpoint was the incidence of Thrombolysis In Myocardial Infarction (TIMI) major bleeding. The secondary

outcome parameter was the composite of major adverse cardiac events (MACE: stent thrombosis, acute coronary syndrome and cardiac death).

### Statistical analysis

Based on a 2.2% rate of stent thrombosis in the group with high platelet aggregation as compared with 0.2% in the group with low platelet aggregation [23], we calculated that 366 patients would provide 80% power to detect significant differences (one-sided alpha value of  $< 0.05$ ). To compensate for potential loss to follow-up, we included 50 additional patients. Normal distribution was tested with the Kolmogorov Smirnov test. Data are expressed as mean, standard deviation (SD), 95% confidence intervals (CIs) median or interquartile range. A ROC curve analysis was used to determine the ability of the tests to distinguish between patients with or without stent thrombosis. The optimal cut-off points were calculated based on the ROC curve to provide the greatest sum of sensitivity and specificity. Statistical comparisons were performed with the *t*-test, the Mann–Whitney *U*-test and the  $\chi^2$ -test when applicable. Kaplan–Meier curves with the Breslow test were used for survival analyses. The Bonferroni correction was used for multiple comparisons. Classification tree analysis (chi-squared automatic interaction detection, CHAID) was used to detect discriminators of the phenotype of clopidogrel response. The analysis included CYP2C19 genotype, common risk factors for coronary artery disease (cigarette smoking, diabetes mellitus, hypertension, family history of coronary artery disease and hyperlipidemia), past medical history (stroke, previous PCI and previous myocardial infarction), co-morbidities (renal failure and periphery or cerebral vascular disease), age, status at hospitalization (stable angina or acute coronary syndrome), concomitant medication (proton pump inhibitors [PPI], calcium channel blockers [CCB] and statins) and sex. Stepwise multivariable logistic regression analysis was used to estimate independent variables responsible for clinical outcome. The multivariate model included: clopidogrel responder status assessed by MEA (ADP + PGE1-induced platelet aggregation), CYP2C19\*2 carrier status, body mass index (BMI), C-reactive protein (CRP) levels, diabetes mellitus, age, renal failure (creatinine clearance  $< 60 \text{ mg mL}^{-1}$ ), myocardial infarction (MI) at admission, sex and use of proton pump inhibitors. All statistical calculations were performed using commercially available statistical software (spss Version 18.0; SPSS Inc., Chicago, IL, USA).

## Results

### Patient demographics

Patient demographics and co-medication are shown in Table 1. Most of the patients underwent non-emergent PCI due to stable angina (66%). Of patients undergoing PCI due to myocardial infarction (34%), one-third presented with symptom onset  $> 48 \text{ h}$ . The majority of patients had high blood pressure and

hyperlipidemia. Almost half of the patients had previous PCI and one-third suffered from previous myocardial infarction (MI). Use of beta-blockers, proton pump inhibitors (PPIs) and statins was high. Five patients (1.2%) were lost to follow-up.

Differences in the demographic data were seen between clopidogrel responders and non-responders. Patients classified as non-responders in the MEA test suffered more frequently from diabetes mellitus (44% vs. 30%;  $P = 0.017$ ), received more frequently emergency PCI due to an acute coronary syndrome (50% vs. 29%;  $P = 0.001$ ), used more often proton pump inhibitors (PPI; 86% vs. 74%;  $P = 0.021$ ), and had higher CRP levels ( $2.4 \text{ mg dL}^{-1}$  vs.  $1.1 \text{ mg dL}^{-1}$ ;  $P < 0.001$ ) and higher platelet counts ( $250 \times 10^9$  vs.  $217 \times 10^9$ ;  $P = 0.002$ ) but had experienced less frequently prior myocardial infarction (21% vs. 37%;  $P = 0.01$ ; Table 1) as compared with clopidogrel responders.

### Performance of different assays for assessment of response to clopidogrel in order to predict stent thrombosis or MACE

Stent thrombosis occurred in 10 patients (2.4%: two acute, five sub-acute and three late). ROC curve analysis demonstrated that platelet aggregation assessed by MEA distinguished between patients with and without subsequent stent thrombosis (ADP + PGE1-induced platelet aggregation, area under the curve = c-index = 0.9, 95% CI = 0.86–0.95,  $P < 0.001$ ; ADP-induced platelet aggregation, c-index = 0.78, 95% CI = 0.63–0.94,  $P = 0.002$ ; Fig. 1A, Table 2) whereas other tests (VASP assay, CPA and PFA100) did not (c-index  $< 0.67$ ;  $P > 0.05$ ; Fig. 1A, Table 1). In accordance, MEA showed higher values for sensitivity and specificity (ADP + PGE1, 90% and 83%; ADP, 70% and 67%) than the VASP assay (70% and 38%), CPA (SC, 90% and 36%; AS, 60% and 42%), PFA100 (70% and 61%) and even the CYP2C19\*2 carrier status (30% and 71%, respectively; Table 2). Although the negative predictive value (the probability of predicting the absence of stent thrombosis) was high for all tests used (93–100%; Table 2), the positive predictive value (the probability of predicting the occurrence of stent thrombosis) was overall low, with the highest value for the ADP + PGE1-induced platelet aggregation by MEA (13% vs. 3–7%; Table 2).

Six stent thromboses occurred in patients presenting with an acute coronary syndrome at admission, whereas four stent thromboses occurred in patients undergoing elective PCI. ROC analysis demonstrated that ADP + PGE1-induced platelet aggregation assessed by MEA distinguished between patients with and without subsequent stent thrombosis in patients presenting with acute coronary syndrome (ACS) as well as in those undergoing elective PCI (ACS, c-index = 0.83, 95% CI = 0.74–0.91,  $P = 0.007$ ; elective PCI, c-index = 0.95, 95% CI = 0.89–1.0,  $P = 0.002$ ; data not shown).

The composite of major adverse cardiac events (MACE: stent thrombosis, acute coronary syndrome and cardiac death) occurred in 52 patients (12.5%). ROC curve analysis demonstrated that platelet aggregation assessed by MEA

**Table 1** Patient demographics

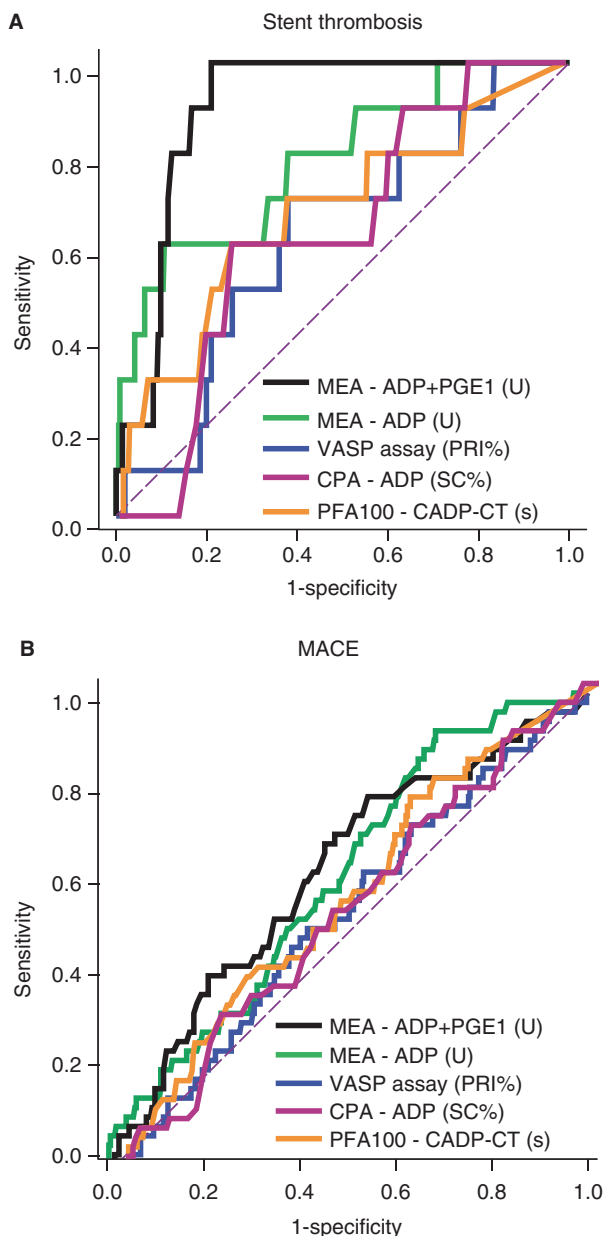
	Whole population <i>n</i> = 416	Clopidogrel non-responder according to MEA ( $\geq 48$ U) <i>n</i> = 81 (20%)	Clopidogrel responder according to MEA (< 48 U) <i>n</i> = 321 (80%)
Age (years)	64 $\pm$ 12	63 $\pm$ 12	64 $\pm$ 12
Gender (male), <i>n</i> (%)	318 (76)	58 (72)	249 (78)
Risk factors/past medical history, <i>n</i> (%)			
Body mass index (BMI; mean $\pm$ SD)	28.1 $\pm$ 5.5	29 $\pm$ 5.8	28 $\pm$ 5.2
Hypertension	352 (84)	66 (83)	275 (86)
Hyperlipidemia	318 (76)	60 (75)	248 (78)
Smoking	230 (55)	46 (58)	174 (55)
Family history of coronary artery disease (CAD)	129 (31)	21 (26)	105 (33)
Diabetes mellitus	135 (32)	36 (44)	97 (30) <sup>†</sup>
Prior percutaneous coronary intervention (PCI)	197 (47)	31 (38)	162 (50)
Prior myocardial infarction	135 (31)	17 (21)	116 (37) <sup>†</sup>
Peripheral arterial occlusive disease	54 (13)	9 (11)	45 (14)
Cerebrovascular disease	41 (10)	7 (9)	34 (11)
Laboratory data (mean $\pm$ SD)			
White blood cell count (WBC; $\times 10^9$ L <sup>-1</sup> )	7.9 $\pm$ 2.6	8.7 $\pm$ 2.5	7.5 $\pm$ 2.5
Platelets ( $\times 10^9$ L <sup>-1</sup> )	224 $\pm$ 71	250 $\pm$ 87	217 $\pm$ 65 <sup>‡</sup>
C reactive protein (mg dL <sup>-1</sup> )	1.3 $\pm$ 1.2	2.4 $\pm$ 0.9	1.1 $\pm$ 1.0 <sup>§</sup>
Hemoglobin (g dL <sup>-1</sup> )	13.3 $\pm$ 1.9	13 $\pm$ 2.0	14 $\pm$ 11.4
Fibrinogen (mg dL <sup>-1</sup> )	413 $\pm$ 119	445 $\pm$ 144	406 $\pm$ 110
Creatinine (mg dL <sup>-1</sup> )	1.3 $\pm$ 0.9	1.4 $\pm$ 0.7	1.3 $\pm$ 1.2
Pre-PCI medications, <i>n</i> (%)			
Aspirin	416 (100)	81 (100)	321 (100)
Clopidogrel	416 (100)	81 (100)	321 (100)
Proton pump Inhibitors (PPI)	317 (76)	69 (86)	236 (74) <sup>†</sup>
$\beta$ blockers	309 (74)	60 (75)	238 (75)
Statins	303 (73)	53 (66)	239 (75)
Angiotensin converting enzyme (ACE) inhibitors	219 (53)	40 (50)	174 (55)
Calcium channel blockers	80 (19)	13 (16)	65 (20)
Medications at discharge (%)			
Aspirin	416 (100)	81 (100)	321 (100)
Clopidogrel	416 (100)	81 (100)	321 (100)
Statins	333 (80)	63 (80)	257 (84)
$\beta$ blockers	334 (80)	65 (83)	256 (84)
PPI	325 (78)	68 (87)	246 (80)
ACE inhibitors	275 (53)	54 (69)	210 (69)
Calcium channel blockers	78 (19)	10 (13)	68 (22)
PCI data			
Elective PCI	274 (66)	41 (50)	229 (71) <sup>‡</sup>
PCI due to an acute coronary syndrome (ACS)	140 (34)	40 (50)	92 (29) <sup>‡</sup>
Non-ST-elevation acute coronary syndrome (NSTEMI-ACS)	67 (16)	19 (48)	49 (53)
ST-elevation myocardial infarction (STEMI)	73 (18)	21 (52)	43 (47)
Number of stents per patient	1.7 $\pm$ 1	1.86 $\pm$ 1.27	1.69 $\pm$ 0.98
Total stent length	31.8 $\pm$ 21.7	33.7 $\pm$ 24.7	31.1 $\pm$ 20.9
CYP2C19 carrier status, <i>n</i> (%)			
*1 (regular metabolizer)	167 (40)	35 (43)	127 (40)
*17 (ultra-metabolizer)	141 (34)	22 (27)	115 (36)
*2 (poor metabolizer)	84 (20)	22 (27)	59 (18)
*2/*17 (mixed metabolizer)	24 (6)	2 (3)	20 (6)

Data are reported as mean  $\pm$  standard deviation (SD), *n* (number of patients) or percentages. MEA, multiple electrode aggregometry.

distinguished between patients with and without subsequent MACE (ADP + PGE1-induced platelet aggregation, c-index = 0.63, 95% CI = 0.55–0.71, *P* = 0.042; ADP-induced platelet aggregation, c-index = 0.62, 95% CI = 0.54–0.70, *P* = 0.039; Fig. 1B), whereas other tests did not (c-index  $\leq$  0.56, *P* > 0.05; Fig. 1B; Table 3).

#### *Incidence of adverse events according to the phenotype and genotype*

The incidence of stent thrombosis was highest among patients classified as poor responders (aggregation  $\geq 48$  U, 12.5%) compared with the regular responders (aggregation 21–47 U,



**Fig. 1.** Receiver operating characteristic (ROC) curves of different assays for prediction of (A) stent thrombosis and (B) the composite of major adverse ischemic events (MACE: acute coronary syndrome, cardiac death and stent thrombosis) during 12 months after stent implantation. VASP, vasodilator-stimulated phosphoprotein phosphorylation assay; PRI, platelet reactivity index; MEA, multiple electrode aggregometry; ADP + PGE1, adenosine diphosphate + prostaglandine E1; CPA, cone and platelet analyzer; SC, surface coverage; PFA100, platelet function analyser; CADP-CT, collagen + adenosine diphosphate-induced closure time.

1%;  $P < 0.001$ ) or the ultra-responders (aggregation  $< 20$  U, 0%;  $P < 0.001$ ; Fig. 2A). The incidence of TIMI major bleeding was numerically highest in ultra-responders (4%), whereas no bleeding events occurred in poor responders (0%,  $P = 0.097$ ; Fig. 2A).

The incidence of stent thrombosis did not differ between regular metabolizers (CYP2C19\*1/\*1, 2.1%), heterozygote

poor metabolizers (CYP2C19\*1/\*2, 3.2%) or homozygote poor metabolizers (CYP2C19\*2/\*2, 0%;  $P = 0.837$ ; Fig. 2B). The incidence of TIMI major bleeding was highest in homozygote ultra-metabolizers (CYP2C19\*17/\*17, 9.5%; Fig. 2C), whereas there was no difference in the incidence of major bleeding between heterozygote ultra-metabolizers (CYP2C19\*1/\*17, 2%) or regular metabolizers (CYP2C19\*1/\*1, 2.9%; Fig. 2C). One stent thrombosis and one major bleeding occurred in mixed metabolizers (diplotypes, CYP2C19\*2/\*17).

#### Correlation between phenotype and genotype in patients suffering from stent thrombosis or major bleeding

When only patients suffering from stent thrombosis were analyzed, platelet aggregation was 3-fold higher in poor metabolizers compared with regular, ultra or mixed metabolizers (mean, 140 U vs. 56 U;  $P < 0.01$ ; Fig. 3, blue circles). In contrast, bleeding events were uniformly distributed between the genotype groups (Fig. 3, red triangles).

#### Survival analysis according to the phenotype and genotype

Kaplan–Meier curves showed an early separation of stent thrombosis and MACE rates between clopidogrel poor responders and clopidogrel responders (12.5% vs. 0.3%,  $P < 0.001$ , Fig. 4A; 18.5% vs. 11.3%,  $P = 0.022$ , Fig. 4C; respectively), whereas poor metabolizers were not at increased risk of developing stent thrombosis or MACE (2.7% vs. 2.5%,  $P = 0.926$ , Fig. 4B; 13.5% vs. 12.1%,  $P = 0.556$ , Fig. 4D; respectively). Although there was a trend toward higher incidences of TIMI major bleeding in ultra-responders vs. regular and poor responders in the MEA test (3.9% vs. 1.8%) or in ultra-metabolizers vs. regular-metabolizers (4.1% vs. 2.2%), neither test was predictive for bleeding events in the survival analysis (Fig. 4E,F), which might be due to an insufficient power of the study to detect significant differences for bleedings.

#### Predictors of clinical events

We used a multiple logistic regression model to estimate independent variables responsible for the occurrence of adverse events (Table 4). The model identified ADP + PGE1-induced platelet aggregation assessed by MEA as an independent predictor of stent thrombosis (OR = 36.9, 95% CI = 4.3–319; Table 4). In contrast, the predictive value of MEA for ACS and MACE lost statistical significance after the inclusion of CRP in the model (Table 4).

#### Combination of geno- and phenotyping data

According to the Standards for Reporting of Diagnostic Accuracy (STARD), we have compared the results of genotyping with those of phenotyping (Fig. 5). From 123 patients with loss of function polymorphism (CYP2C19\*2), only 33

**Table 2** Statistical estimates for the prediction of stent thrombosis by different assays for assessment of responsiveness to clopidogrel

Test	Stent thrombosis (definite and probable) <i>n</i> = 10 (2.4%)							
	c-index (95% CI)	<i>P</i>	Cut-off	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %	Positive likelihood ratio
MEA: ADP-PGE1 (U)	0.90 (0.86–0.95)	< 0.001	48	90	83	13	100	3.5
MEA: ADP (U)	0.78 (0.63–0.94)	0.002	46	70	67	7	100	2.1
VASP assay (% PRI)	0.62 (0.46–0.79)	0.204	42	70	38	3	98	1.1
PFA100: CADP-CT (s)	0.66 (0.48–0.84)	0.084	105	70	61	4	98	1.8
CPA: ADP (SC%)	0.62 (0.47–76)	0.205	4.6	90	36	3	98	1.4
CPA: ADP (AS $\mu\text{m}^2$ )	0.45 (0.25–0.65)	0.606	43	60	42	3	98	1.0
CYP2C19 *2	0.56 (0.32–0.69)	0.950	*2/*2	30	71	3	93	1.0
			*1/*2					

VASP, vasodilator-stimulated phosphoprotein phosphorylation assay; PRI, platelet reactivity index; MEA, multiple electrode aggregometry; ADP + PGE1, adenosine diphosphate + prostaglandine E1; CPA, cone and platelet analyzer; SC, surface coverage; AS, average size of platelet aggregates; PFA100, platelet function analyser; CADP-CT, collagen + adenosine diphosphate-induced closure time; c-index, area under the curve in the receiver operating characteristics (ROC).

**Table 3** Statistical estimates for the prediction of MACE (stent thrombosis (ST), acute coronary syndrome (ACS) and cardiac death) by different assays for assessment of responsiveness to clopidogrel

Test	MACE (ST, ACS, cardiac death) <i>n</i> = 52 (12.5%)							
	c-index (95% CI)	<i>P</i>	Cut-off	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %	Positive likelihood ratio
MEA: ADP-PGE1 (U)	0.63 (0.55–0.71)	0.042	48	30	81	20	88	1.6
MEA: ADP (U)	0.62 (0.54–0.70)	0.039	46	50	64	17	89	1.4
VASP assay (%PRI)	0.54 (0.45–0.63)	0.38	42	68	37	14	88	1.1
PFA100: CADP-CT (s)	0.56 (0.48–0.64)	0.062	105	44	62	15	88	1.2
CPA: ADP (SC%)	0.54 (0.38–0.62)	0.38	4.6	72	36	14	89	1.1
CPA: ADP (AS $\mu\text{m}^2$ )	0.53 (0.45–0.61)	0.47	43	60	43	14	88	1.1
CYP2C19 *2	0.48 (0.39–0.57)	0.63	*2/*2	33	71	14	87	1.2
			*1/*2					

VASP, vasodilator-stimulated phosphoprotein phosphorylation assay; PRI, platelet reactivity index; MEA, multiple electrode aggregometry; ADP + PGE1, adenosine diphosphate + prostaglandine E1; CPA, cone and platelet analyzer; SC, surface coverage; AS, average size of platelet aggregates; PFA100, platelet function analyser; CADP-CT, collagen + adenosine diphosphate-induced closure time; c-index, area under the curve in the receiver operating characteristics (ROC).

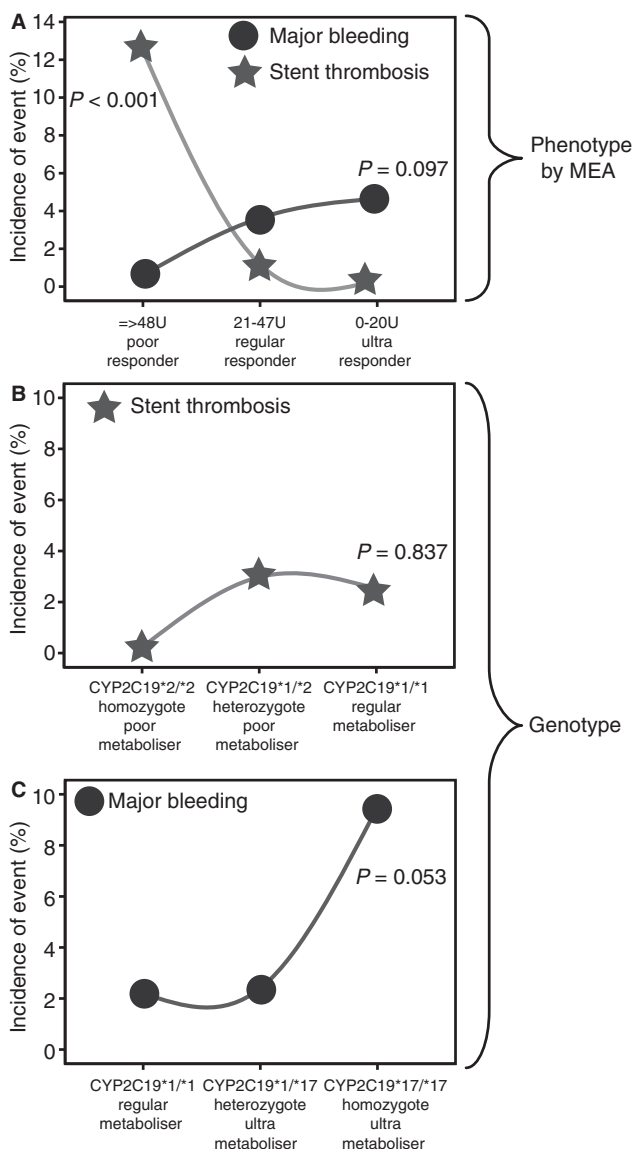
patients (27%) were classified as clopidogrel poor responders in the MEA assay (Fig. 5, right column: abnormal test result in both tests). This highlights that genotype predicted phenotype only in one of four patients. From the study population of patients with an abnormal test result in both assays (poor metabolizer in the genotyping study, CYP2C19\*2; poor responder in the phenotyping study, aggregation  $\geq$  48 U), three patients (9%) developed an event during a clinical follow-up (Fig. 5, right column). This is lower than the incidence of stent thrombosis when using only results of the phenotyping study by MEA (12.5%; Fig. 2A). Therefore, double testing did not improve the prediction of occurrence of stent thrombosis.

From 279 patients without a loss of function polymorphism (CYP2C19\*1), 231 (83%) were simultaneously classified as clopidogrel responders in the MEA assay (Fig. 5, left column: normal result in both tests). In the study population of patients with a normal test result in both assays (regular metabolizer in

the genotyping study, CYP2C19\*1; responder in the phenotyping study, aggregation < 48 U) one event occurred (0.4%). This is comparable to the incidence of stent thrombosis when using only results of the MEA assay (0.3%; Fig. 2A). Therefore, double testing did not improve the prediction of absence of stent thrombosis.

#### *Contribution of clinical characteristics and CYP2C19 genotype to the phenotype of the response to clopidogrel*

Classification tree analysis (CHAID) was used to detect discriminators of the phenotype of clopidogrel response. The analysis included common CYP2C19 genotype, risk factors for coronary artery disease, past medical history, co-morbidities, co-medication, age, status at hospitalization (stable angina or acute coronary syndrome) and sex. Acute coronary syndrome at hospitalization emerged as the strongest variable influencing

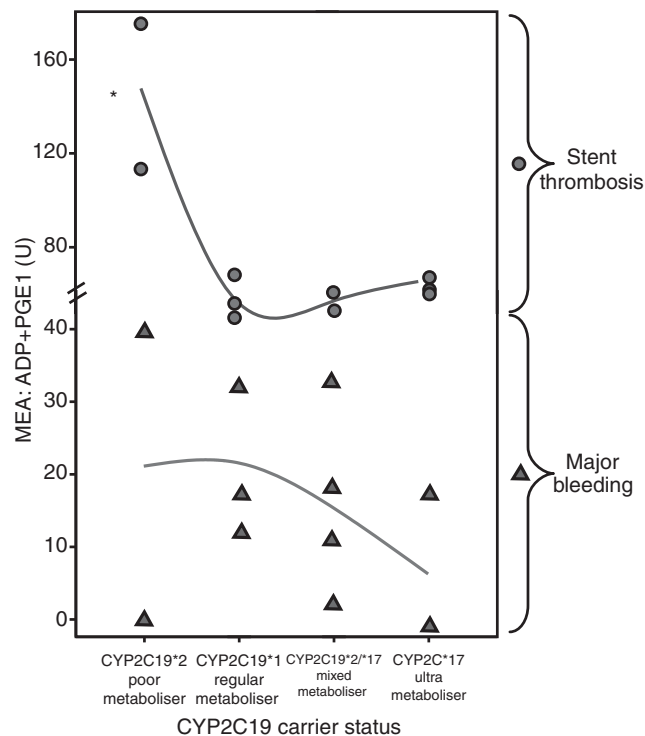


**Fig. 2.** Incidence of adverse events according to the ADP + PGE1-induced platelet aggregation assessed by multiple electrode aggregometry (MEA; 0–20 U = ultra responder, 21–47 U = responder, ≥ 48 U = poor responder) and genotype (CYP2C19\*1/\*1 = regular metabolizer; CYP2C19\*1/\*2 = heterozygote poor metabolizer; CYP2C19\*2/\*2 = homozygote poor metabolizer; CYP2C19\*1/\*17 = heterozygote ultra-metabolizer; CYP2C19\*17/\*17 = homozygote ultra-metabolizer).

clopidogrel response status (Fig. 6): the frequency of patients classified as poor-responders reached 29% in this group. The second strongest discriminator was diabetes mellitus (24% poor responders). Neither genotype nor other clinical characteristics influenced the phenotype of the response to clopidogrel in this analysis.

**Discussion**

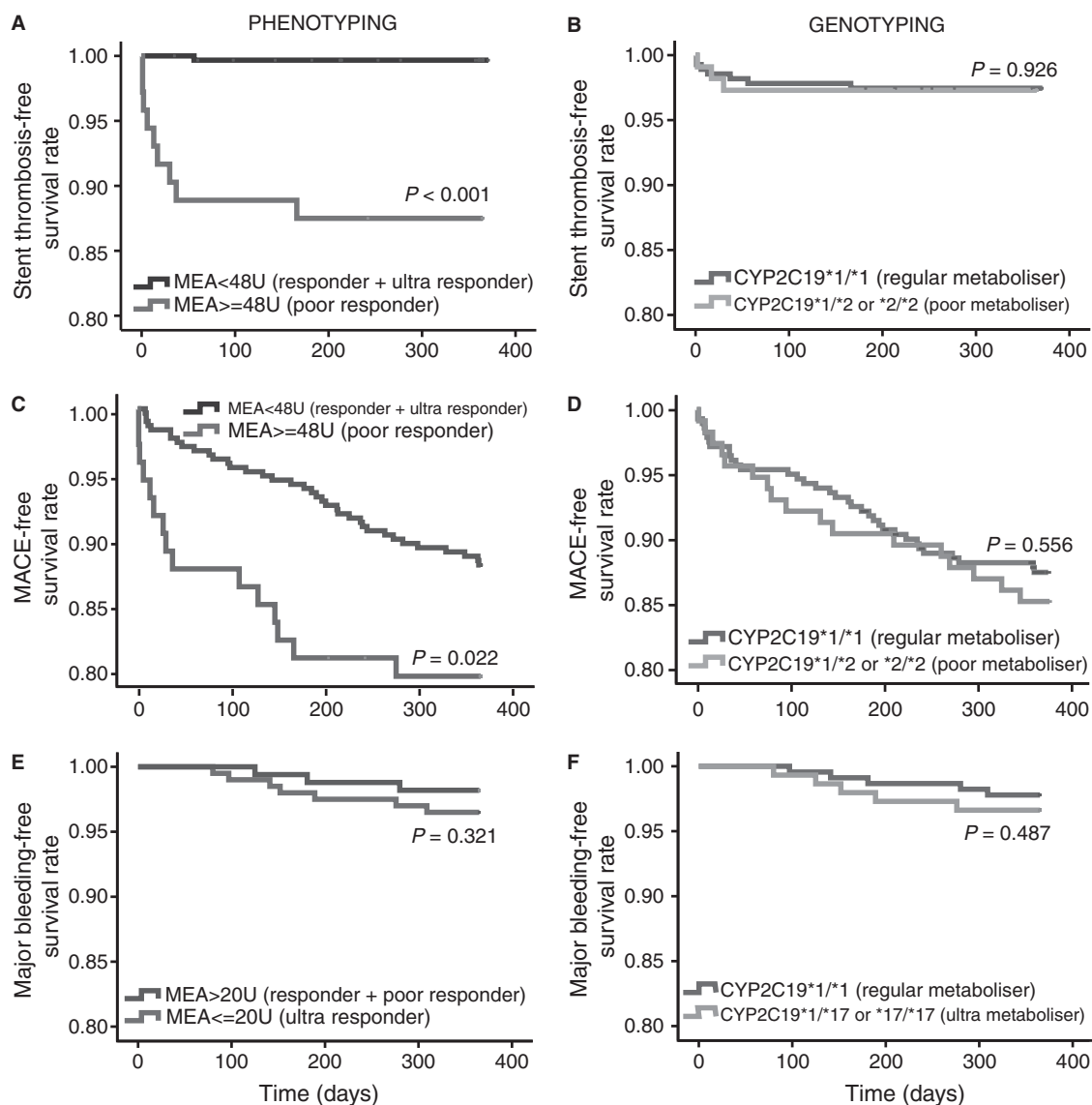
The central finding of this head to head comparison of laboratory approaches used for assessment of the antiplatelet



**Fig. 3.** Correlation between CYP2C19 carrier status (CYP2C19\*1 = regular metabolizer; CYP2C19\*2/\*17 = mixed metabolizer; CYP2C19\*2 = poor metabolizer; CYP2C19\*17 = ultra-metabolizer) and ADP + PGE1-induced platelet aggregation in patients suffering from adverse events. Blue circle = stent thrombosis; red triangle = major bleeding; \*P < 0.01.

effect of clopidogrel is that phenotyping of clopidogrel effect by MEA independently predicted stent thrombosis during 1-year follow-up. Although predictive values for tests assessing the phenotype of clopidogrel effect [23,33] and genotype of the CYP2C19 allele have been shown in independent studies [24,25], our study provides the first direct comparisons between the pheno- and genotyping with regard to bleeding and ischemic events. Genotyping of the CYP2C19\*2 allele predicted the phenotype of clopidogrel effect only in 27% of patients in our study. This finding is in line with previously published data showing that the CYP2C19\*2 carrier status accounted only for up to 12% of the variability in the platelet response to clopidogrel in multivariate analyses [10,34], thus suggesting that other variables like unknown genetic variants or clinical characteristics contribute to this phenomenon. In our analysis diabetes mellitus and PCI for acute coronary syndrome independently affected response to clopidogrel, which confirms previous findings [16,35,36]. Likewise, these clinical parameters are also predisposing factors for occurrence of stent thrombosis [37]. Other investigations have shown that age, BMI, co-medication, renal failure and reduced left ventricular function also contribute to the reduction in clopidogrel effect [6,7,10,16,35], thus implicating its multifactorial nature. Therefore, better performance of functional platelet assays like MEA might be due to the fact that factors



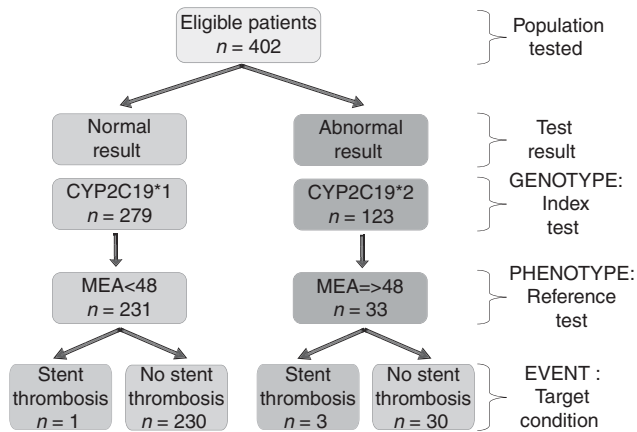


**Fig. 4.** Kaplan–Meier estimates of events of the efficacy and safety outcomes in relation to the responder status assessed by multiple electrode aggregometry (MEA; ADP + PGE1) and CYP2C19 genotypes (\*2 or \*17).

**Table 4** Incidence of clinical endpoints and the results of the Cox regression analysis

Endpoints, <i>n</i> (%)	Whole population <i>n</i> = 416	Clopidogrel non-responders according to MEA ( $\geq 48$ U) <i>n</i> = 81 (20%)	Clopidogrel responders according to MEA (< 48 U) <i>n</i> = 321 (80%)	Univariate COX regression OR (95% CI)	Multivariate COX regression OR (95% CI)
Major adverse cardiac events (MACE)	52 (12.5)	15 (21)	37 (12)	1.9 (1.02–3.4)*	1.67 (0.86–3.2)
Acute coronary syndrome (ACS)	41 (9.8)	13 (17)	27 (9)	2.2 (1.2–4.3)*	1.8 (0.85–3.8)
Stent thrombosis	10 (2.4)	9 (12.5)	1 (0.3)	40 (5–315)**	36.9 (4.3–319)**
Cardiac death	20 (4.8)	6 (8)	14 (5)	1.8 (0.7–4.8)	2.1 (0.7–6.2)
TIMI major bleeding	11 (2.6)	0 (0)	11 (4)	0.036 (0–33)	0 (0–infinity)

The multivariate model included: clopidogrel responder status assessed by MEA (ADP + PGE1-induced platelet aggregation), CYP2C19\*2 carrier status, body mass index (BMI), C-reactive protein (CRP) levels, diabetes mellitus, age, renal failure (creatinine clearance < 60 mg mL<sup>-1</sup>), myocardial infarction (MI) at admission, sex and use of proton pump inhibitors. Major adverse cardiac events: stent thrombosis, acute coronary syndrome and cardiac death. \* $P < 0.001$ ; \*\* $P < 0.05$



**Fig. 5.** Flow diagram showing diagnostic accuracy of the CYP2C19\*2 carrier status compared with platelet aggregation assessed by multiple electrode aggregometry (MEA) according to the Standards for Reporting of Diagnostic Accuracy (STARD). Incidence of stent thrombosis is given for patients having the same test result in both tests simultaneously.

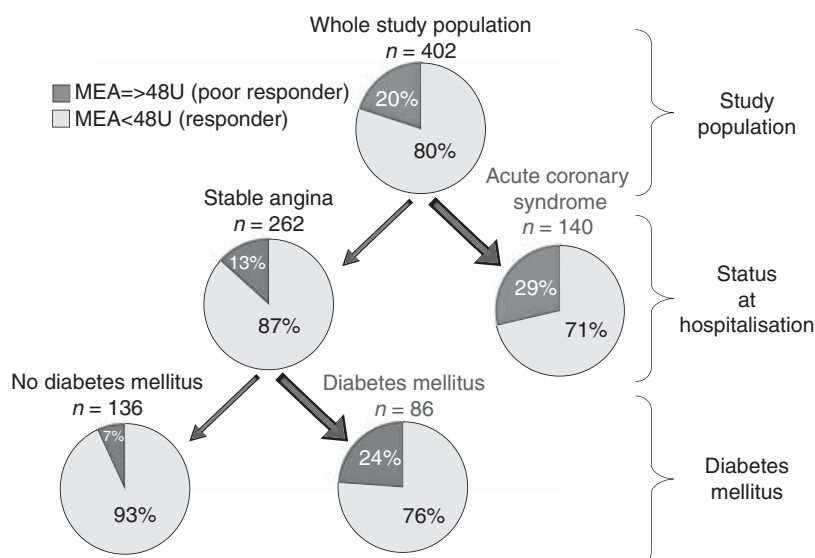
affecting response to clopidogrel are similar to those predicting stent thrombosis [37].

Our results showing no impact of the loss-of-function allele on the primary efficacy outcome in the survival analysis are in line with the results of the PLATO, CURE and ACTIVE A trials [38,39], where the effect of clopidogrel was similar in patients who were heterozygous or homozygous for loss-of-function alleles and in those who were not carriers of the alleles [38]. In contrast, the TRITON-TIMI trial [40] and other studies showed opposite findings [11,13,25,41]. In the TRITON-TIMI trial patients with the CYP2C19\*2 polymorphism had a 3-fold higher risk of developing stent thrombosis compared with patients without the loss-of-function polymorphism [40]. In another study, CYP2C19\*2 carrier status was associated with a

6-fold increased risk of stent thrombosis [11]. Indeed, taking into consideration only patients presenting with stent thrombosis in our study, a very good agreement was found between genotyping and phenotyping. This might be a reason why genetic profiling in patients presenting with stent thrombosis (information given after the event) provides significant data [42]. Nevertheless, the predictive value of genotyping must be confirmed in randomized double-blind trials [43]. Currently, several studies are underway to evaluate the association between genetic profiling and platelet response to clopidogrel (SPICE, ACCEL-2C19, ACCELAMI2C19 and PAPI-2) [44]. A large observational, open-label study is examining genotype-guided comparison of clopidogrel vs. prasugrel in CYP2C19\*2 carrier for cardiovascular outcomes in patients with acute coronary syndrome (GeCCO). As both genotyping and phenotyping have been shown to provide complementary information to stratify risk [45], both methods are used in the TARGET-PCI study: a randomized open label study to guide antiplatelet therapy. However, randomized double-blind trials with use of pharmacogenetic profiling to guide antiplatelet drug regimen are to our knowledge not registered yet.

A possible explanation for a lack of association between the CYP2C19\*2 polymorphism and stent thrombosis might be drug–drug interactions with clopidogrel as use of proton pump inhibitors and calcium channel blockers, which might interfere with clopidogrel metabolism and therefore influence patient’s outcome was high in our study [6,7].

In terms of bleeding events, neither genotyping of the CYP2C1\*17 allele nor phenotyping of the clopidogrel effect by various assays was predictive for bleeds during long-term clinical follow-up, which might be due to the low event rates. Concerning the CYP2C1\*17 polymorphism, our finding is in line with the CURE and ACTIVE A trials showing that the effect of clopidogrel on bleeding did not vary according to



**Fig. 6.** Chi-squared automatic interaction detection (CHAID) analysis for response status to clopidogrel assessed by multiple electrode aggregometry (MEA).

genotypic subgroups [38]. Although in the PLATO trial the gain-of-function CYP2C1\*17 allele corresponded to a higher incidence of major bleedings (11.5% vs. 9.5%), the interaction between treatment and genotype groups was not significant [39]. In accordance, although the frequency of major bleedings was higher in CYP2C19\*17/\*17 carriers compared with non-carriers (9.5% vs. 2%) in our study, the CYP2C1\*17 polymorphism was not associated with bleeds in the survival analysis. Unlike our results, another observational study showed that CYP2C19\*17 carrier status was associated with enhanced response to clopidogrel, which corresponded to an increased risk of bleeding [46].

As the \*2 and \*17 alleles are not randomly associated, they represent a linkage disequilibrium [10,47]. Therefore, CYP2C19\*17 carriers are less likely to carry the \*2 allele and *vice versa* [48]. Indeed, in our study population none of the patients was homozygous on both \*2 and \*17 loci but 6% were diplotypes: heterozygous on \*17 and \*2 (mixed metabolizers). However, the impact of this allele on antiplatelet effect of clopidogrel and patients' outcome remains unclear as one stent thrombosis and one major bleeding occurred in those patients. This observation is in line with another study, where diplotypes showed high variability in platelet function [45].

Performance of MEA to predict stent thrombosis (c-index, 0.9) was higher than for prediction of MACE (c-index, 0.63). Similar values for prediction of MACE were reported in another study comparing assays used to phenotype the response to clopidogrel: light transmittance aggregometry, VerifyNow, PFA-100, InnovancePFA-100, CPA and Plateletworks, (c-index, 0.50–0.63) [22], which is lower than the common threshold to denote a test as useful (c-index, 0.8). In accordance, moderate values for sensitivity and specificity have been shown for those assays (sensitivity, 55–63%; specificity, 29–64%) [22], which are lower than the values for MEA shown in our study (sensitivity, 90%; specificity, 83%). This indicates that a global test like MEA might be a better assay for risk assessment of stent thrombosis, a clinical endpoint with most interest when considering clopidogrel non-responsiveness.

Although the incidence of stent thrombosis is decreasing in the era of new drug-eluting stents and novel platelet inhibitors [49–51], clopidogrel is currently the only authorized agent in patients undergoing elective PCI [52]. Secondly, clopidogrel will probably still be used in some countries due to an economic impact since clopidogrel generics have entered into the market. Furthermore, recent studies in patients suffering from an acute coronary syndrome suggest that high platelet reactivity also occurs in patients treated with prasugrel. Two studies showed that up to 25% of individuals did not achieve the required platelet inhibition by prasugrel, which correlated with higher rates of stent thrombosis [53,54]. In line with this, randomized trials in chronic hemodialysis patients who were clopidogrel non-responders indicated that 12–19% of them were also prasugrel non-responders [55,56]. In this context, studies like ours aiming to compare different laboratory approaches for prediction of adverse events in non-responders to ADP receptor inhibitors are important.

### Limitations

The results might be influenced by chance based on a limited sample size, indicating that while our results are interesting the definite conclusion can not be deducted. Nevertheless, the comparison of genotype and outcome data with other reports indicates that our study sample was representative. CYP2C19 allele frequencies as well as the incidence of stent thrombosis and bleedings were consistent with those reported in large clinical trials [25,38,39,57]. Therefore, our findings should provide reliable information about the interactions of the CYP2C19 polymorphism, and the levels of platelet response to clopidogrel assessed by functional assays with outcomes during long-term treatment of patients managed invasively. Accordingly, concerns have been raised regarding personalized antiplatelet therapy in low-risk patients in the GRAVITAS and the TRIGGER-PCI trials. In the GRAVITAS trial a high clopidogrel maintenance dose (150 mg) vs. standard clopidogrel dose (75 mg) was not associated with a reduction of MACE [58]. The TRIGGER-PCI trial has been stopped because the trial would not have sufficient power to deliver significant results; it compared prasugrel vs. clopidogrel in patients with an insufficient response to clopidogrel suffering from a stable coronary artery disease. Interestingly, the event rates for the composite endpoint were 5-fold higher in our study compared with GRAVITAS or TRIGGER-PCI, which might be a reason why testing of platelet function was predictive for events in our study. The difference in the event rates might be due to several reasons. Firstly, the duration of follow-up was 2-fold longer in our study (12 vs. 6 months). Secondly, randomization was performed 12–24 h after PCI in the GRAVITAS trial and 2–7 h after clopidogrel maintenance dose intake the day after successful PCI in the TRIGGER-PCI trial. Therefore, it is possible that patients experiencing events early after PCI or those with unsuccessful or complicated PCI procedures were excluded from both trials. Thirdly, in both trials mostly second-generation drug-eluting stents were used whereas first-generation drug-eluting stents were implanted in our study. Fourthly, we also included patients with ST-elevation myocardial infarction, who had the highest rate of events (17%), indicating that patients at high risk could mostly benefit from personalized antiplatelet treatment.

A further limitation is a variable interval between clopidogrel intake and blood sampling. Therefore, lack of correlation between geno- and phenotyping might be due to the fact that blood was sampled immediately after stent placement in our study. PCI leads to the release of multiple coagulation mediators, which additionally activate platelets through transient 'by-passing' signalling pathways [59]. Hence, it is possible that measurement of platelet aggregation directly after stent placement does not solely reflect the response to clopidogrel but rather shows the overall platelet reactivity. Nevertheless, the values obtained after PCI showed the best correlation with stent thrombosis in our study. As clopidogrel reached steady state in 80% of patients in our study at the time point of blood

sampling, it is also unlikely that this factor would significantly influence results. Moreover, data are lacking regarding which time-point of blood sampling is most appropriate for prediction of events.

## Conclusion

The PEGASUS-PCI study shows that phenotyping of platelet response to clopidogrel by MEA might be a good risk predictor for stent thrombosis. The good performance of MEA was also confirmed when compared with genotyping of the CYP2C19\*2 allele or with other tests assessing the phenotype of clopidogrel effect. Nevertheless, as our findings are exploratory, we do not recommend any assay to guide the antiplatelet treatment in the routine clinical practice until this strategy is confirmed in properly powered randomized trials.

## Addendum

J.M. Siller-Matula: conception and study design, collection of laboratory data, analysis and interpretation of data, manuscript drafting. G. Delle-Karth: collection of laboratory data, manuscript drafting. I.M. Lang: conception and study design, collection of laboratory data, manuscript drafting. T. Neunteufl: collection of laboratory data, manuscript drafting. M. Kozinski: analysis and interpretation of data, manuscript drafting. J. Kubica: analysis and interpretation of data, manuscript drafting. G. Maurer: manuscript drafting. K. Linkowska: genetic analyses, manuscript drafting. T. Grzybowski: genetic analyses, manuscript drafting. B. Jilma: conception and study design, analysis and interpretation of data, manuscript drafting. All authors contributed to the revisions of the draft and approval of the final manuscript.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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