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Routine Assessment of On-Clopidogrel Platelet Reactivity and Gene Polymorphisms in Predicting Clinical Outcome Following Drug-Eluting Stent Implantation in Patients With Stable Coronary Artery Disease

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Objectives This study sought to assess the usefulness of clopidogrel-pathway genotyping and on-treatment platelet reactivity (OTR) testing in predicting major adverse cardiac events (MACE) in stable coronary artery disease (CAD) patients receiving drug-eluting stents (DES) under dual antiplatelet (clopidogrel plus aspirin) therapy.

Background The role of pharmacogenetics and OTR in predicting MACE—death, myocardial infarction, or stent thrombosis—in stable CAD patients scheduled for DES implantation is still debated.

Methods Patients with stable CAD treated by DES implantation (n = 1,432) were genotyped with a TaqMan OpenArray (Applied Biosystems, Carlsbad, California) and assessed for OTR with the VerifyNow $P2Y_{12}$ test (Accumetrics Inc., San Diego, California). Genes tested were *ABCB1, CYP1A2, CYP2B6*9, CYP2C8*3, CYP2C9*2, CYP2C19, CYP3A4, CYP3A5*3, P2RY12*, and *PON1CYP2C19*. High OTR was defined as $P2Y_{12}$ reaction units $\geq$230. The endpoint at 12-month follow-up was MACE occurring during antiplatelet therapy.

Results All groups that were stratified for loss-of-function variants of the cytochrome P450 gene *CYP2C19* had significant hazard ratios (HR) for MACE (genotypic HR: 1.41, 95% confidence interval [CI]: 1.06 to 1.89, p = 0.01; allelic HR: 1.56, 95% CI: 2.26 to 1.2, p = 0.01). Variants of other clopidogrel-pathway genes were not significantly associated with MACE. When OTR was assessed, clinical significance was found only in high-risk diabetic (HR: 2.11, 95% CI: 1.29 to 3.45, p < 0.001) and chronic kidney disease (HR: 2.03, 95% CI: 1.03 to 4.02, p = 0.04) patients.

Conclusions *CYP2C19* metabolizer status is an independent predictor of MACE after DES implantation and can be used for prognostication in all stable CAD patients. High OTR, as assessed by the VerifyNow $P2Y_{12}$ test, is an independent predictor of MACE only for high-risk subsets, that is, patients with diabetes or chronic kidney disease. (J Am Coll Cardiol Intv 2013;6:1166–75) © 2013 by the American College of Cardiology Foundation

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Clopidogrel is administered with aspirin for dual antiplatelet therapy of coronary artery disease (CAD) (1). Despite the proven efficacy of clopidogrel, interindividual variability in drug action significantly influences therapeutic outcome (2,3). In fact, being a prodrug, clopidogrel requires modification into a thiol metabolite within the liver before acquiring full activity (4), so the efficacy of clopidogrel's antiaggregating effect depends on pharmacodynamic factors related to drug-metabolizing enzymes, such as the cytochrome P450 (CYP) family members (e.g., CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP3A5) and paraoxonase 1 (PON1), the transport molecule mediating the drug's uptake by intestinal cells (i.e., adenosine triphosphatase–binding cassette, subfamily B [MDR/TAP], member 1, or ABCB1), and its molecular target on platelets (i.e., the purinergic receptor P2Y, G-protein coupled, 12, or $P2Y_{12}$) (5–8).

Following percutaneous revascularization, the rate of major adverse cardiac events (MACE), such as stent thrombosis, myocardial infarction, or death, is significantly increased in those patients presenting with high on-treatment platelet reactivity (OTR) (3,9,10). Currently, there are 2 major approaches to identifying high-risk patients: the first assesses the metabolizing status, which involves genotyping to uncover single-nucleotide polymorphisms (SNP) that have a negative impact on the bioactivation of clopidogrel; the other entails the response status and refers to functional phenotypic testing to measure residual platelet reactivity in peripheral whole blood. However, it is not yet clear which is the more reliable method of MACE prognostication. We therefore decided to evaluate these 2 approaches for predicting the occurrence of 12-month MACE following deployment of drug-eluting stents (DES) in patients with stable CAD and on dual antiplatelet therapy.

Methods

Study population. All consecutive patients scheduled between January 7, 2008 and January 31, 2010 to undergo elective DES implantation at the Clinica Mediterranea (Naples, Italy) were assessed for their suitability for the study. Exclusion criteria were: either non–ST-segment or ST-segment elevation myocardial infarction; cardiogenic shock; allergy/intolerance to aspirin and/or clopidogrel; ongoing serious bleeding or bleeding diathesis; platelet count $\leq$75.000/mm^3; planned or undelayable noncardiac surgery; previous percutaneous coronary intervention or coronary artery bypass grafting; severe liver disease (e.g., cirrhosis or portal hypertension); and life expectancy <1 year due to other medical conditions. All enrolled patients gave informed consent prior to the index procedure. Diabetes mellitus (DM) was diagnosed according to current guidelines (11). Chronic kidney disease (CKD) was defined as an estimated glomerular filtration rate <60 ml/min/1.73 m^2 (12). This study was approved by the local ethics committees.

Stenting procedure and pharmacological approach. At the discretion of the operator, either intraprocedural unfractionated heparin (goal-activated clotting time >250 s) or bivalirudin was used before the procedure. Tirofiban (a glycoprotein IIb/IIIa inhibitor) was administered according to the operator's judgment. All patients received at least 1 DES. Either first-generation (Cypher Select [Cordis, Johnson & Johnson, Miami Lakes, Florida]; Taxus Libertè [Boston Scientific Corporation, Natick, Massachusetts]) or second-generation (Endeavor Resolute or Resolute Integrity [Medtronic CardioVascular, Santa Rosa, California]; Promus or Promus Element [Boston Scientific]; Nobori [Terumo, Tokyo, Japan]) DES were implanted. Patients received aspirin (100 mg daily) and clopidogrel (75 mg daily) before stent deployment. In patients not already on therapy, a clopidogrel loading dose of 600 mg was administered the day before the procedure.

Post-procedure management and follow-up. Dual antiplatelet therapy, consisting of the administration of aspirin (100 mg/day) and clopidogrel (75 mg/day) for at least 12 months, was prescribed to all patients. In patients deemed at high risk (including those with DM, CKD, multivessel CAD, chronic total occlusion, or bifurcation lesions), clopidogrel administration was recommended for more than 12 months. Clinical follow-up was performed via a visit to the outpatient clinic or by a telephone interview with the patient at 1 month and, thereafter, every 3 months until the 12th month. If clopidogrel was discontinued before the recommended period, the date of discontinuation was recorded.

Platelet reactivity assay. The inhibitory effect of clopidogrel on platelet aggregation was measured with the VerifyNow $P2Y_{12}$ test (Accumetrics Inc., San Diego, California) (13) within 24 h of DES deployment. Specifically, the test was performed in all instances on the morning following DES implantation, within 3 h of the last maintenance dose of clopidogrel. In patients receiving periprocedural tirofiban, the test was performed at least 48 h after drug discontinuation. OTR was assessed after, rather than before, stent implantation for the following reasons: 1) it was not the purpose of the present study to assess the relationship between OTR and periprocedural myocardial infarction; 2)

Abbreviations and Acronyms

CAD = coronary artery disease
CKD = chronic kidney disease
CYP = cytochrome P450
DES = drug-eluting stent(s)
DM = diabetes mellitus
HR = hazard ratio(s)
LVEF = left ventricular ejection fraction
MACE = major adverse cardiac events
OTR = on-treatment platelet reactivity
$P2Y_{12}$ = purinergic receptor P2Y, G-protein coupled, 12
PRU = $P2Y_{12}$ reaction units
SNP = single-nucleotide polymorphism

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knowledge of high OTR would have influenced the operator's decision to use glycoprotein IIb/IIIa inhibitors and/or an additional loading dose of clopidogrel; and 3) it would have led to the direct exclusion of patients treated with balloon angioplasty rather than with stent implantation.

The VerifyNow $P2Y_{12}$ test is a rapid, point-of-care, cartridge-based, platelet-function assay designed to directly measure the blockade of drugs on the $P2Y_{12}$ receptor (13). The level of platelet aggregation is measured as an increase in light transmittance as activated platelets bind and aggregate fibrinogen-coated beads. A proprietary algorithm is used to report values in $P2Y_{12}$ reaction units (PRU). A PRU $\geq$230 reflects greater platelet reactivity and was used to define patients with high OTR (10). In a secondary analysis, a PRU >208 was also used to define patients with high OTR (14).

DNA extraction, selection of SNP, and genotyping. After DES deployment, 4 ml whole peripheral blood was obtained from patients, the genomic deoxyribonucleic acid (DNA) extracted from mononuclear cells with the DNA QIAamp Midi kit (Qiagen Inc., Valencia, California) according to the manufacturer's instructions, and the DNA stored at −20°C until used. The quantity and quality of the genomic DNA were verified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) before being assayed with the TaqMan OpenArray Genotyping System (Applied Biosystems, Carlsbad, California). The clopidogrel-metabolizing pathway SNPs used in the customized genotyping panel were: *ABCB1* (rs1045642); *CYP1A2* (rs762551); *CYP2B6*9* (rs3745274); *CYP2C8*3* (rs 10509681); *CYP2C9*2* (rs1799853); *CYP2C19*2* (rs42 44285); *CYP2C19*3* (rs4986893); *CYP2C19*4* (rs28399504); *CYP2C19*5* (rs56337013); *CYP2C19*17* (rs12248560); *CYP3A4* (rs2242480, rs4986910); *CYP3A5*3* (rs776746); *P2RY12* (rs2046934); and *PON1* (rs854560, rs662) (15,16). Sample processing was fully automated using the Freedom EVO150 robotic workstation (Tecan Group Ltd., Männedorf, Switzerland). Fluorescent signals were recorded by the OpenArray instrument, and specific cluster parameters were automatically obtained to precisely assign the relative genotypes. All samples were genotyped in duplicate to verify the results and avoid technical errors. All patients were stratified according to genotype effect on clopidogrel metabolism (extensive, intermediate, and poor metabolizers). For *CYP2C19*, patients were classified as: 1) extensive metabolizers, for individuals not carrying a loss-of-function variant (**1*1*) or for carriers of at least 1 increased enzymatic-activity allele (**1*17* or **17*17*); 2) intermediate metabolizers, for carriers of 1 loss-of-function allele (**1*2*, **1*3*, **1*4*, or **1*5*); 3) poor metabolizers, for carriers of 2 loss-of-function alleles (**2*2, *2*3, *2*4,*2*5*, *3*3, *3*4, *3*5*, *4*5*, etc.); or 4) unknown metabolizers, for patients presenting with 1 loss-of-function allele plus a **17* allele.

Study endpoints. The primary endpoint of the study was on-treatment MACE, defined as cardiac death, nonfatal myocardial infarction, or stent thrombosis during clopidogrel treatment. Therefore, each patient was censored once off clopidogrel. Myocardial infarction during follow-up was defined as any typical change in cardiac biomarkers in conditions associated with cardiac ischemia, according to American College of Cardiology guidelines (17). Stent thrombosis was defined according to Academic Research Consortium criteria (18). An independent medical committee assigned and recorded all occurrences of MACE. MACE rate was stratified according to OTR and genotype.

Statistical analyses. The choice of sample size was based on the assumptions of: 1) an annual MACE rate >10%; and 2) a high OTR occurrence in 30% of patients. These assumptions were derived from the data available in the literature (14,19–21). Continuous selected variables are given as mean ± SD. Normality assumption was verified graphically (i.e., QQ plot) and was confirmed using the Shapiro-Wilk test. Categorical variables are expressed in percentages. Comparisons between groups were performed by Student *t* test or chi-square test. The percentage of successfully genotyped samples and the average genotyping success rate for each SNP (>85%) were calculated; Hardy-Weinberg equilibrium was determined using the chi-square goodness-of-fit test. Initially, to examine the effects of the selected SNP and of PRU $\geq$230, *t* test and 1-way analysis of variance were employed. Survival curves were generated using Kaplan-Meier product limit estimator. Unadjusted comparisons between predicted functional groups defined by PRU $\geq$230 versus <230 or by genotyping (e.g., *CYP2C19*-based metabolizer status) and the composite MACE outcomes were compared using log-rank test. The presence of at least 1 of the following criteria identified patients at high risk: DM; CKD; age >75 years; multivessel disease; and left ventricular ejection fraction (LVEF) <40% (22–30). Cox proportional hazards model was used to provide hazard ratios (HR) with 95% confidence intervals and adjustment for previously selected clinical predictors (selected a priori by the clinician according to the features of our patient population and to $p < 0.01$ at univariate analysis), including: age; LVEF; sex; DM; smoking; number of diseased coronary arteries; DES type; estimated glomerular filtration rate; chronic total occlusion; and CKD. To correct for multiple testing, 1,000 bootstrap iterations were computed. The area under the receiver-operating characteristic curve (AUC) was finally used to determine the discriminatory ability of the model. AUC is the probability that a randomly selected patient with the event had a predicted probability of event higher than that of a randomly selected patient without the event. A value of 1 denotes perfect discrimination, whereas a value of 0.5 is no better than chance. For all tests, $p<0.05$ was considered

statistically significant. Statistical analyses were performed with the Stata program (version 11/SE, StataCorp LP, College Station, Texas).

Results

Study population. A complete clinical follow-up was available for 1,432 of 1,461 (98%) patients. MACE at follow-up occurred in 114 (7.9%) patients. Specifically, cardiac death occurred in 14 (0.9%) patients, nonfatal myocardial infarction in 49 (3.4 %), and stent thrombosis in 51 (3.5%) patients (definite 1.0%, n = 14; probable 1.7%, n = 25; possible 0.8%, n = 12). Clinical, angiographic, and procedural features of patients with MACE (MACE group) and without MACE (event-free group) are summarized in Tables 1 and 2. The MACE group had higher percentages of individuals with LVEF <40%, CKD, DM, or chronic total occlusion than did the event-free group. The majority of patients (89.3%) in the 2 groups received second-generation DES. The median duration of dual antiplatelet therapy was 20 (range 15 to 26) months. Dual antiplatelet therapy lasted >1 year in 70% of the MACE group and in 69.2% of the event-free group (p = 0.87).

Response status and metabolizing status as MACE predictors within the whole population. As assessed by the VerifyNow $P2Y_{12}$ test, 40.5% (584 of 1,432) of patients had a PRU ≥230. No significant interactions were observed between response status and other drugs metabolized by cytochrome P450 (namely, statins, proton-pump inhibitors, and calcium-channel blockers). Durations of dual antiplatelet therapy were 20 (range 13.5 to 26.5) months in patients with PRU ≥230 and 19 (range 12 to 25) months in patients with PRU <230 (p = 0.15). Genotyping was successful in 1,432 patients (>85%). Intermediate metabolizers represented 27% of the total population, whereas poor metabolizers comprised 4.7%. In detail, the high-OTR group (i.e., with a PRU ≥230) was composed of 28.3% intermediate metabolizers and 6.7% poor metabolizers. On the contrary, the low-OTR group (PRU <230) was composed of 27.5% intermediate metabolizers and 3.5% poor metabolizers. The analysis of *CYP2C19* genotype distribution revealed a higher percentage of poor metabolizers in the MACE group (Table 3). A significant association with absolute PRU values was observed in *CYP2C19* metabolizer classes at the genotypic (1-way analysis of variance, p < 0.02) and allelic (1-tailed student *t* test, p < 0.001)

Table 1. Clinical Features at Baseline of the Global and MACE-Stratified Populations

	Global (n = 1,432)	Event-Free Group (n = 1,318)	MACE Group (n = 114)	p Value
General characteristics				
Age, yrs	64.9 ± 10.2	64.8 ± 10.2	65.3 ± 9.8	0.67
Body mass index, kg/m^2	27.9 ± 4.3	27.9 ± 4.4	27.5 ± 3.7	0.24
Male	1,107 (77.7)	1,024 (77.7)	83 (72.8)	0.24
Current smokers	385 (26.9)	354 (26.8)	31 (27.2)	0.34
LVEF, %	52.4 ± 8.45	52.5 ± 8.37	51.5 ± 9.35	0.31
Blood biochemistry				
Cholesterol, mg/dl	161.2 ± 43.9	161.5 ± 43.9	162.2 ± 43.9	0.43
High-density lipoprotein, mg/dl	52.2 ± 28.0	52.3 ± 28.4	48.7 ± 27.0	0.10
Low-density lipoprotein, mg/dl	88.0 ± 37.7	87.7 ± 38.1	91.5 ± 35.9	0.16
Triglycerides, mg/dl	109.3 ± 73.1	108.6 ± 73.4	116.3 ± 70.8	0.14
Therapy				
Clopidogrel MD	379 (26.5)	355 (27.0)	24 (21.0)	0.15
Statins	1,260 (87.9)	1,164 (88.3)	96 (84.2)	0.08
Proton-pump inhibitor	693 (48.4)	639 (48.5)	54 (47.3)	0.40
Calcium-channel blocker	358 (25.0)	330 (25.0)	28 (24.5)	1.00
Glycoprotein IIb/IIIa inhibitor	12 (0.8)	12 (0.9)	0.0	0.61
Comorbidities				
LVEF <40%	153 (10.7)	131 (9.9)	22 (19.3)	0.01
CKD	332 (23.2)	289 (21.9)	43 (37.7)	<0.001
Diabetes mellitus	572 (39.9)	511 (38.7)	61 (53.5)	<0.001
Hypertension	1,048 (73.2)	966 (73.2)	82 (71.9)	0.73
MVD	1,006 (70.2)	912 (69.2)	94 (82.4)	<0.001

Values are mean ± SD or n (%). Clopidogrel maintenance dose refers to patients already treated with clopidogrel before drug-eluting stent implantation. In patients not already on therapy, a clopidogrel loading dose of 600 mg was administered the day before the procedure.

CKD = chronic kidney disease; LVEF = left ventricular ejection fraction; MACE = major adverse cardiac events; MD = maintenance dose; MVD = multivessel disease.

Table 2. Angiographic and Procedural Features of the Global and MACE-Stratified Populations

	Global (n = 1,432)	Event-Free Group (n = 1,318)	MACE Group (n = 114)	p Value
Second-generation DES type	1,279 (89.3)	1,204 (91.3)	75 (65.8)	0.17
Stent length, mm	28.2 ± 12.3	28.0 ± 12.4	27.5 ± 11.2	0.31
Stents per patient	1.45 ± 0.79	1.44 ± 0.78	1.53 ± 0.92	0.63
Complex lesion, B2/C	855 (59.7)	787 (59.7)	68 (59.6)	0.86
Chronic total occlusion	156 (10.9)	136 (10.3)	20 (17.5)	0.01
Bifurcation lesion	281 (19.6)	258 (19.6)	23 (20.2)	0.88
Minimal lumen diameter, mm				
Pre-procedure	0.51 ± 0.39	0.51 ± 0.38	0.52 ± 0.3	0.96
Post-procedure	3.24 ± 0.57	3.25 ± 0.57	3.19 ± 0.51	0.28
Reference vessel diameter, mm				
Pre-procedure	3.12 ± 0.56	3.13 ± 0.56	3.03 ± 0.48	0.08
Post-procedure	3.29 ± 0.54	3.29 ± 0.54	3.28 ± 0.40	0.78
Maximal inflation pressure, atm	19.0 ± 4.96	19.0 ± 4.92	19.1 ± 5.40	0.89
Balloon-to-artery ratio	1.05 ± 0.16	1.05 ± 0.13	1.06 ± 0.35	0.51

Values are n (%) or mean ± SD.
DES = drug-eluting stent(s); MACE = major adverse cardiac events.

levels. No significant differences were found between the MACE group and the event-free group when OTR and the other analyzed SNPs of the clopidogrel pathway were considered (Table 3).

Table 4 reports log-rank estimates for the phenotypic and genetic tests. Consistent associations at the genotypic and allelic levels were found only for *CYP2C19* variants; in fact, log-rank test for MACE-free survival showed higher MACE rates in poor and intermediate metabolizers with respect to extensive metabolizers (Table 4, Figs. 1A and 1B). No significant association was observed between MACE and high OTR or the other tested SNPs (Table 4, Fig. 1C). No significant association was found when considering a different PRU cutoff (≥208) (Online Table 1).

Univariate and multivariate Cox proportional hazards models were used to provide corrected HRs for both the response status and the metabolizing status. The following independent variables were included in the Cox regression as potential factors affecting composite outcome: age; sex; smoking; DES type; DM; CKD; LVEF <40%; and

Table 3. Summary of Distributions of Patients According to OTR and SNP

Test	Comparison	Event-Free Group (n = 1,318)	MACE Group (n = 114)	p Value	p, Allele
OTR*	Low vs. high OTR	778/540 (59/41)	70/44 (61/39)	0.63	
CYP2C19 variants†	EM vs. IM vs. PM	855/347/58 (68/27/5)	64/39/10 (57/34/9)	0.02	<0.001
ABCB1 (3,435 C>T)	CC vs. CT vs. TT	346/597/275 (28/49/23)	24/56/26 (23/53/24)	0.44	0.28
*CYP1A2*1F* (164 A>C)	AA vs. AC vs. CC	577/516/155 (46/41/13)	44/50/16 (40/45/15)	0.44	0.21
*CYP2B6*9* (516 G>T)	GG vs. GT vs. TT	602/424/98 (53/38/9)	56/39/6 (55/39/6)	0.63	0.47
*CYP2C8*3* (416 G>A)	GG vs. GA vs. AA	948/267/36 (76/21/3)	88/20/3 (79/18/3)	0.70	0.44
*CYP2C9*2* (430 C>T)	CC vs. CT vs. TT	986/236/16 (80/19/1)	92/15/1 (85/14/1)	0.38	0.18
CYP3A4 (20,239 G>A)	GG vs. GA vs. AA	898/232/18 (78/20/2)	76/23/3 (74/23/3)	0.48	0.28
CYP3A4 (1,334 T>C)	TT vs. TC vs. CC	1235/8/0 (99/1/0)	109/0/0 (100/0/0)	0.40	0.40
*CYP3A5*3* (6,986 A>G)	AA vs. AG vs. GG	1096/140/6 (88/11/1)	91/18/1 (83/16/1)	0.22	0.10
P2RY12 (744 T>C H1H2)‡	TT vs. TC vs. CC	1041/204/6 (83/16/1)	89/20/1 (81/18/1)	0.72	0.49
PON1 (260 T>A)	TT vs. TA vs. AA	521/534/159 (43/44/13)	43/47/13 (42/46/12)	0.94	0.92
PON1 (672 A>G)	AA vs. AG vs. GG	599/547/103 (48/44/8)	57/43/9 (52/40/8)	0.66	0.50

The p values were calculated with Pearson statistic. p, Allele are p values obtained in allelic status. High = $P2Y_{12}$ reaction units ≥230. Low = $P2Y_{12}$ reaction units <230. *On-treatment platelet reactivity, assessed by the VerifyNow $P2Y_{12}$ test. †Composite of *CYP2C19* variants: *CYP2C19*2* (681 G>A), *CYP2C19*3* (636 G>A), *CYP2C19*4* (1 A>G), *CYP2C19*5* (1,297 C>T), and *CYP2C19*17* (806 C>T). ‡H1H2 haplotype (H2 minor haplotype) tagged by rs2046934, rs10935838, rs5853517, and rs6809699.
EM = extensive metabolizers; IM = intermediate metabolizers; MACE = major adverse cardiac events; OTR = on-treatment platelet reactivity; PM = poor metabolizers; SNP = single nucleotide polymorphism.

Table 4. Log-Rank Test for All CAD Patients

Test	Comparison	p Value	p, Allele
OTR*	Low vs. high	0.08	
CYP2C19 variants†	EM vs. IM vs. PM	0.01	<0.00
ABCB1 (3,435 C>T)	CC vs. CT vs. TT	0.11	0.70
*CYP1A2*1F* (–164 A>C)	AA vs. AC vs. CC	0.40	0.39
*CYP2B6*9* (516 G>T)	GG vs. GT vs. TT	0.73	0.70
*CYP2C8*3* (416 G>A)	GG vs. GA vs. AA	0.48	0.40
*CYP2C9*2* (430 C>T)	CC vs. CT vs. TT	0.24	0.10
CYP3A4 (20,239 G>A)	GG vs. GA vs. AA	0.16	0.08
CYP3A4 (1,334 T>C)	TT vs. TC vs. CC	0.12	0.20
*CYP3A5*3* (6,986 A>G)	AA vs. AG vs. GG	0.29	0.14
P2RY12 (744 T>C H1H2)‡	TT vs. TC vs. CC	0.68	0.67
PON1 (260 T>A)	TT vs. TA vs. AA	0.97	0.97
PON1 (672 A>G)	AA vs. AG vs. GG	0.71	0.71

The p values were calculated by log-rank for genetic analysis and adjusted for bootstrap sampling method. p, Allele are p values obtained in allelic status. *On-treatment platelet reactivity, assessed by the VerifyNow $P2Y_{12}$ test. †Composite of *CYP2C19* variants: *CYP2C19*2* (681 G>A), *CYP2C19*3* (636 G>A), *CYP2C19*4* (1 A>G), *CYP2C19*5* (1,297 C>T), and *CYP2C19*17* (806 C>T). ‡H1H2 haplotype (H2 minor haplotype) tagged by rs2046934, rs10935838, rs5853517, and rs6809699.

CAD = coronary artery disease; other abbreviations as in Table 3.

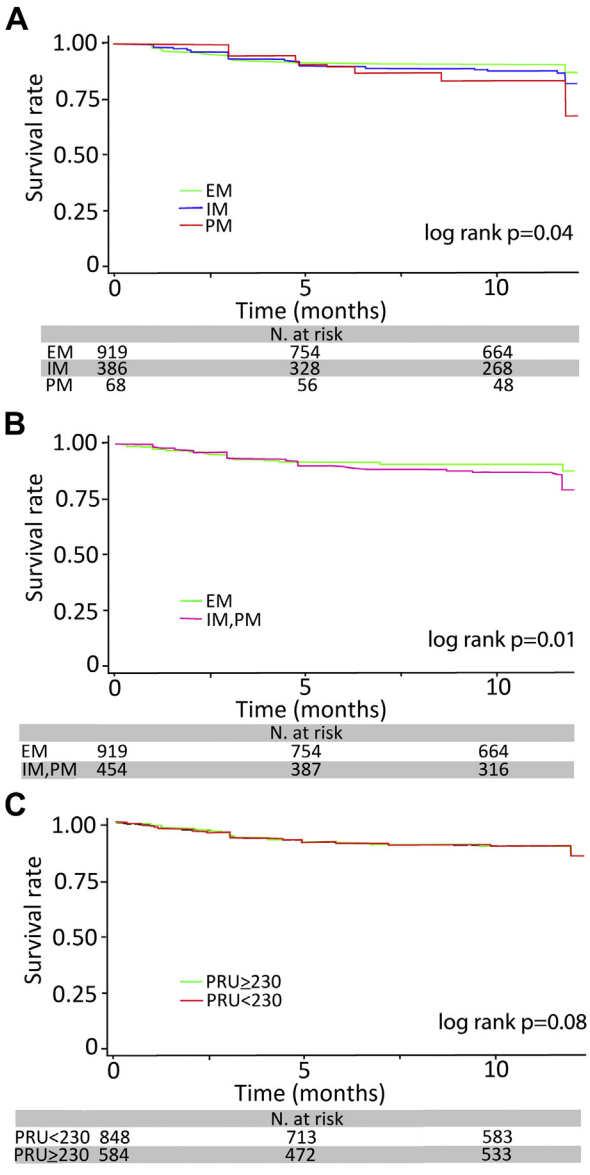

Figure 1. Kaplan-Meier Curves for MACE-Free Survival

Patients were stratified based on *CYP2C19* metabolizer status **(A),** allelic level **(B),** or on-treatment platelet reactivity **(C)**. EM = extensive metabolizers; IM = intermediate metabolizers; MACE = major adverse cardiac events; PM = poor metabolizers; PRU = $P2Y_{12}$ reaction units.

multivessel disease. Treatments with statins, proton-pump inhibitors, and/or calcium-channel blockers were not included because the role as clinical predictors was not significant.

We found that MACE rate was higher in carriers of loss-of-function *CYP2C19* alleles (patients classed as either intermediate or poor metabolizers) than in noncarriers (extensive metabolizer patients) (Table 5). The HR computed for the other analyzed SNP did not reveal significant differences for MACE occurrence (Online Tables 2 and 3). There was no significant difference in MACE rates when patients were stratified according to the PRU cutoff (Table 5).

To evaluate the prognostic ability of both the response status and the metabolizing status, multivariate logistic regression was undertaken first with clinical predictors alone and then including both genetic and OTR variables. AUC was 0.682 when considering only clinical predictors, slightly increased (0.701, p = 0.18) when genetic assay predictors were included, and unchanged (0.683, p = 0.51) with OTR assay predictor. Results identical to those of the whole population were found after excluding patients who received tirofiban (Online Tables 4 to 7).

Response status and metabolizing status as MACE predictors in subgroups at high risk. The predictive accuracies of the OTR and of *CYP2C19*-based metabolizer stratification were further assessed with the log-rank test in the high-risk subgroups of patients (i.e., those with DM, CKD, age >75 years, multivessel disease, or LVEF<40%). To obviate the low effect size caused by low frequency of composite cardiovascular events, *CYP2C19*-based categories were considered only in allelic status. The p values were calculated for each analyzed class (phenotypic and genetic). *CYP2C19*-based metabolizer stratification was confirmed to have prognostic value for MACE in all high-risk groups (Table 6, Figs. 2A and 2B). However, the OTR-based assay had prognostic value only for diabetic and CKD patients (Table 6, Figs. 3A and 3B). Similarly, univariate and multivariate Cox regression exhibited significant HR values for *CYP2C19*- and OTR-based assays in patients

Table 5. Univariate and Multivariate Cox Proportional Hazard Ratios for OTR- and *CYP2C19*-Based Predictive Tests in All Patients

		Univariate Model			Multivariate Model		
	Comparison	HR	95% CI	p Value	HR	95% CI	p Value
OTR*	Low vs. high	1.02	0.7–1.49	0.90	1.34	0.84–2.11	0.20
CYP2C19 variants†	EM vs. IM vs. PM	1.41	1.06–1.89	0.01	1.38	1.01–1.91	0.04
	EM vs. (IM + PM)	1.56	1.26–2.26	0.01	1.58	1.04–2.38	0.03

For the adjusted p values, bootstrap sampling method (1000 reps) was employed. *On-treatment platelet reactivity, assessed by the VerifyNow $P2Y_{12}$ test. †Composite of *CYP2C19* variants: *CYP2C19*2* (681 G>A), *CYP2C19*3* (636 G>A), *CYP2C19*4* (1 A>G), *CYP2C19*5* (1,297 C>T), and *CYP2C19*17* (806 C>T).

CI = confidence interval(s); HR = hazard ratio(s); other abbreviations as in Table 3.

copresenting with DM or CKD (Table 7). Potential interaction effects evaluated in a subset population were not significant.

In diabetic patients, the AUCs were 0.642 with clinical predictors, 0.665 (p = 0.68) with metabolizer predictors, and 0.652 (p = 0.73) with the OTR. In CKD patients, the AUC was 0.717 for clinical predictors, 0.748 (p = 0.47) when including *CYP2C19* genotyping variables, and 0.727 (p = 0.78) for the phenotypic predictors. The prognostic values of both response and metabolic status in diabetic patients according to the treatment (insulin versus no insulin), and in CKD patients according to the presence of DM, are reported as supplementary data (Online Table 8), as is the impact of second-generation DES (Online Tables 9 to 11).

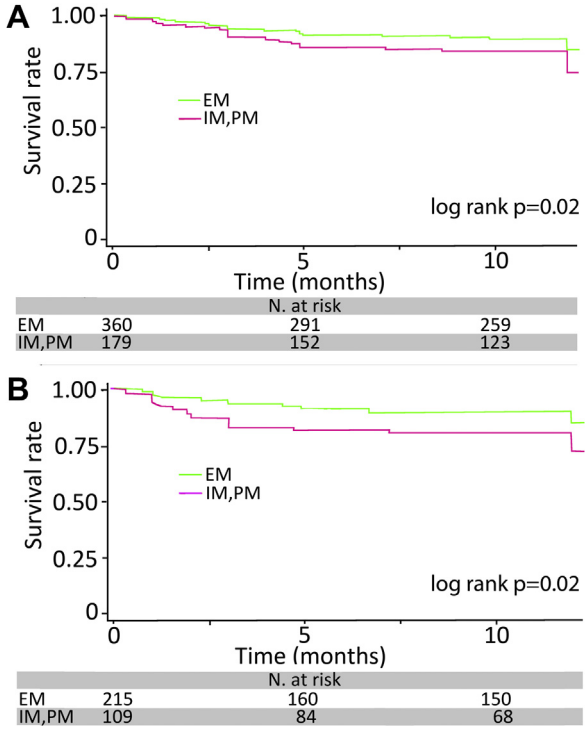

Figure 2. Kaplan-Meier Curves of MACE-Free Survival for *CYP2C19* Metabolizer Groups

Survival in patients copresenting with diabetes mellitus **(A)** or chronic kidney disease **(B)**. Abbreviations as in Figure 1.

Discussion

Two major findings of the present study applying to patients with stable angina are: 1) routine assessment of *CYP2C19* polymorphisms can be used to risk-stratify stable CAD patients receiving DES; and 2) routine assessment of OTR with the VerifyNow $P2Y_{12}$ test has a predictive value only in high-risk (diabetic or CKD) patients.

Prognostication of MACE with genotyping. The present study strengthens the concept that the genetic status of *CYP2C19* can be used to predict the occurrence of MACE after stenting in stable CAD patients (6,7,31–34). In agreement with Mega et al. (35), the results of the current study support the notion that carrying at least 1 loss-of-function *CYP2C19* allele increases the risk of MACE. Indeed, intermediate and poor metabolizer categories of patients were found singularly or in a combined manner (allelic status) to be associated with a higher occurrence of MACE during clopidogrel therapy (7,35). *CYP2C19* is a highly polymorphic gene that is critical for 2 essential oxidative steps of clopidogrel bioactivation (4). The clinical consequences of decreased clopidogrel activation due to deleterious effects of *CYP2C19* variants have been extensively described (6,31–33). In addition, our study also agrees with others reporting that polymorphisms in *ABCB1*, *CYP1A2*, *CYP2B6*9*, *CYP2C8*, *CYP2C9*2*, *CYP3A4*, *CYP3A5*3*, *P2RY12*, or *PON1* are not statistically associated with increased risk of MACE (6,36–38).

Table 6. Log-Rank Test in High-Risk Subsets of Patients

	High OTR*	*CYP2C19* Variants†
Diabetes mellitus	0.01	0.02
CKD	0.03	0.02
Age >75 yrs	0.81	0.03
Multivessel disease	0.53	0.04
LVEF <40%	0.96	0.04

*On-treatment platelet reactivity, assessed by the VerifyNow $P2Y_{12}$ test ($P2Y_{12}$ reaction units ≥230). †Composite of *CYP2C19* variants: *CYP2C19*2* (681 G>A), *CYP2C19*3* (636 G>A), *CYP2C19*4* (1 A>G), *CYP2C19*5* (1,297 C>T), and *CYP2C19*17* (806 C>T).

Abbreviations as in Tables 1 and 3.

Prognostication of MACE with the VerifyNow $P2Y_{12}$ test. Our data extend and clarify the current knowledge on the potential clinical benefit of assessing OTR in patients scheduled for elective DES implantation. When dealing with stable CAD patients with an expected overall low

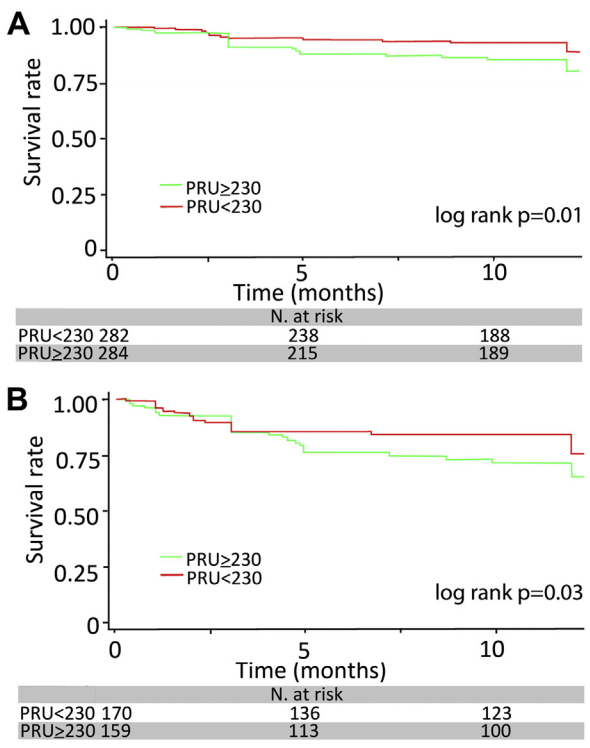

Figure 3. Kaplan-Meier Curves of MACE-Free Survival Stratified for Clopidogrel Reactivity

Survival in diabetic patients **(A)** and chronic kidney disease patients **(B)** stratified into phenotypic groups based on the VerifyNow $P2Y_{12}$ test. Abbreviations as in Figure 1.

MACE rate, the assessment of OTR (due to its modest sensitivity and specificity) is unlikely to have a prognostic value (14,39). On the contrary, when dealing with the high-risk population (including stable patients with DM and/or CKD, and patients with acute coronary syndromes), the assessment of OTR may have a relevant clinical benefit (40–43). This interpretation explains why, for example, in the ADAPT-DES (Assessment of Dual Antiplatelet Therapy with Drug-Eluting Stents) trial, the OTR (assessed with the VerifyNow $P2Y_{12}$ test) was a strong independent predictor of stent thrombosis at 30 days only in patients with acute coronary syndromes but not in patients with stable CAD (39). This result has been recently confirmed (44). The POPULAR (Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI) trial demonstrated a modest accuracy of 4 platelet reactivity tests (including the VerifyNow $P2Y_{12}$ test) in predicting clinical outcome (43). Possible explanations for the discrepancy between our study and the POPULAR trial are: 1) differences in the risk at baseline of the patient populations (as suggested by the different rate of DM, CKD, and bifurcation lesions); and 2) differences in the type of stent used in the 2 studies. Indeed, the extensive use of second-generation DES in our study may have had a relevant role in reducing the MACE rate at follow-up. As recently reported, when compared with first-generation DES, second-generation DES are associated with a 43% lower risk of definite stent thrombosis and with a 23% lower risk of death for up to 2 years (45). Finally, our interpretation may also explain the negative findings of some recent trials that aimed to demonstrate a clinically relevant effect of an antiplatelet therapy tailored according to the OTR result. Indeed, the strategies of increasing the clopidogrel maintenance dose (14) and of switching to prasugrel (46,47) in patients with high OTR failed to improve clinical outcome.

Table 7. Univariate and Multivariate Cox Proportional Hazard Regression for OTR- and *CYP2C19*-Based Predictive Tests in High-Risk Patients

		Univariate Model			Multivariate Model		
	High-Risk Subset	HR	95% CI	p Value	HR	95% CI	p Value
High OTR*	Diabetes mellitus	2.11	1.29–3.45	<0.00	2.40	1.48–4.20	0.03
	CKD	2.03	1.03–4.02	0.04	2.09	1.01–4.67	0.04
CYP2C19 variants†	Diabetes mellitus	1.98	1.08–3.64	0.02	1.45	1.01–3.12	0.01
	CKD	1.55	1.06–2.27	0.02	2.00	1.21–3.10	<0.00

For the adjusted p values, bootstrap sampling method (1,000 reps) was employed. *On-treatment platelet reactivity, assessed by the VerifyNow $P2Y_{12}$ test (P2Y12 reaction units ≥230). †Composite of *CYP2C19* variants: *CYP2C19*2* (681 G>A), *CYP2C19*3* (636 G>A), *CYP2C19*4* (1 A>G), *CYP2C19*5* (1,297 C>T), and *CYP2C19*17* (806 C>T).

Abbreviations as in Tables 1, 3, and 5.

Study limitations. The results of the present study should be limited to the boundaries of the functional assay used (i.e., the VerifyNow $P2Y_{12}$ test) and cannot be directly extended to other clinical assays proposed to assess OTR (48,49). At present, there is no standardized procedure, due to significant variability among the methods and the absence of uniform cutoff values for identifying patients at higher risk. In addition, the optimal timing for platelet function testing (that is, whether on admission, just before or after surgery, or weeks after discharge) has not been assessed. Some evidence does exist on the superiority of measuring platelet function at 1 month after intervention (50), but other trials are required to better clarify the position of routine assessment of platelet assays in clinical practice. Lack of exact timing between the last clopidogrel maintenance dose and the VerifyNow test assessment may represent a further limitation. However, it is unlikely that a <3-h interval may have had an impact on the VerifyNow test result. The results in high-risk patients represent a subgroup analysis of a registry. Therefore, this finding should be tested in specifically designed randomized controlled trials. Moreover, the observed 7.9% MACE rate was lower than that hypothesized; this might make our study slightly underpowered. However, the observed MACE rate was in the range of the 95% confidence interval of the expected incidence (adjusted Wald interval:

3.6% to 13%). The baseline and procedural characteristics of our patient cohort may explain the high stent thrombosis rate. Compared with those in other studies, our cohort had greater incidences of DM, CKD, and bifurcation lesions. Finally, the cost-effectiveness of routine genotyping and residual platelet function testing procedures, as well as their therapeutic impact, were not studied and should be further investigated.

Conclusions

Routine assessment of *CYP2C19* polymorphisms can be used to risk stratify stable CAD patients receiving DES, whereas routine assessment of OTR with the VerifyNow $P2Y_{12}$ test has a predictive value only in high-risk stable CAD patients, such as those with DM or CKD. Randomized controlled trials are warranted to address whether: 1) antiplatelet therapy guided by genetic testing demonstrates a clinically relevant effect in all patients with stable CAD treated by DES implantation; and 2) antiplatelet therapy guided by OTR testing alone or in combination with genetic testing has a clinically relevant effect in diabetic and CKD patients with stable CAD treated by DES implantation.

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Key Words: angioplasty ■ clopidogrel ■ genetics ■ negative outcome ■ platelet reactivity ■ predictive test.

APPENDIX

For supplementary tables, please see the online version of this paper.