Genotyping of six clopidogrel-metabolizing enzyme polymorphisms has a minor role in the assessment of platelet reactivity in patients with acute coronary syndrome

María Henar García-Lagunar, Luciano Consuegra-Sánchez, Pablo Conesa- Zamora**, Javier Ruiz-Cosano***, Federico Soria Arcos*, Luis García de Guadiana**, Pedro Cano Vivar*, Juan Antonio Castillo-Moreno*, Antonio Melgarejo-Moreno**

Departments of Hospital Pharmacy, *Cardiology, **Clinical Analysis, ***Pathology
Santa Lucía General University Hospital (HGUSL); Cartagena-*Spain*

Abstract

Objective: To evaluate the contribution of six polymorphisms to the platelet reactivity in patients with acute coronary syndrome (ACS) treated with clopidogrel.

Methods: Cross-sectional study of 278 consecutive patients with ACS. Detailed clinical information for each patient was collected and genotypes (*CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C19*17*, *CYP3A4*1B*, and *PON1*-Q192R) were evaluated with TaqMan® and KASPar® assays. Platelet reactivity was measured with VerifyNow®.

Results: Mean age of patients was 66±11 years and 182 (65.5%) patients presented ACS without ST-segment elevation. A total of 206 (74.1%) patients presented poor response to clopidogrel (PRC). *CYP2C19*2* polymorphism (p=0.038) was associated with PRC in the univariate setting. In the multiple logistic regression analysis, the risk factors for PRC were the presence of *CYP3A4*1B* allele (odds ratio [OR] 4.03; 95% confidence interval [CI] 1.01–16.34), age (OR 1.43; 95% CI 1.03–2.00), and body mass index (OR 4.05; 95% CI 1.21–13.43), whereas elevated hemoglobin was a protective factor. Discrimination of PRC through the model that included the six polymorphisms added modest information to the model based on clinical variables (C statistic difference 3.9%).

Conclusion: *CYP3A4*1B* allele may be an independent determinant of PRC in patients with ACS, although the variability in response to clopidogrel explained by the six polymorphisms is poor when compared to clinical variables. *(Anatol J Cardiol 2017; 17: 303-12)*

Keywords: acute coronary syndrome; clopidogrel; platelet; aggregometry; polymorphism

Introduction

Although the current guidelines for acute coronary syndrome (ACS) give preference to ticagrelor and prasugrel, a lot of ACS patients continue to receive clopidogrel as medical treatment (1). However, despite dual antiplatelet therapy, a large number of patients present incomplete platelet inhibition (2, 3), and a high residual platelet reactivity on clopidogrel, also termed poor response to clopidogrel (PRC), is associated with increased cardiovascular ischemic events and an unfavorable prognosis (4). Mechanisms contributing to PRC are not entirely well known and are probably multifactorial (5–7).

Clopidogrel is a prodrug that requires biotransformation to generate an active metabolite. It is metabolized by the hepatic cytochrome P450 (CYP1A2, CYP2B6, and CYP2C19) and transformed into the intermediate metabolite, 2-oxo-clopidogrel, which is further oxidized by various isoenzymes (CYP2B6, CYP2C9, CYP2C19, and CYP3A4) and paraoxonase 1 (*PON1*) into an inactive carboxyl group and a highly unstable active thiol derivative (Fig. 1) (8).

To date, there have been few studies (9–15) that evaluate a potential association between *CYP3A4*1B* allele and response to clopidogrel. These studies (9–12, 14, 15) were conducted in healthy subjects (15), in patients with stable coronary artery disease (10), those undergoing elective percutaneous coronary intervention (9, 12), patients with a history of stent thrombosis (11), or mixed patient populations with stable and unstable coronary artery disease (14). Only one previous study (13) has been

Address for correspondence: María Henar García Lagunar, MD, Department of Hospital Pharmacy
Santa Lucía General University Hospital, Cartagena-*Spain*
Phone: +0034968128600 (ext 951485) Fax: +0034 968128628 E-mail: henargl@gmail.com
Accepted Date: 22.11.2016 **Available Online Date:** 01.02.2017

DOI:10.14744/AnatolJCardiol.2016.7390

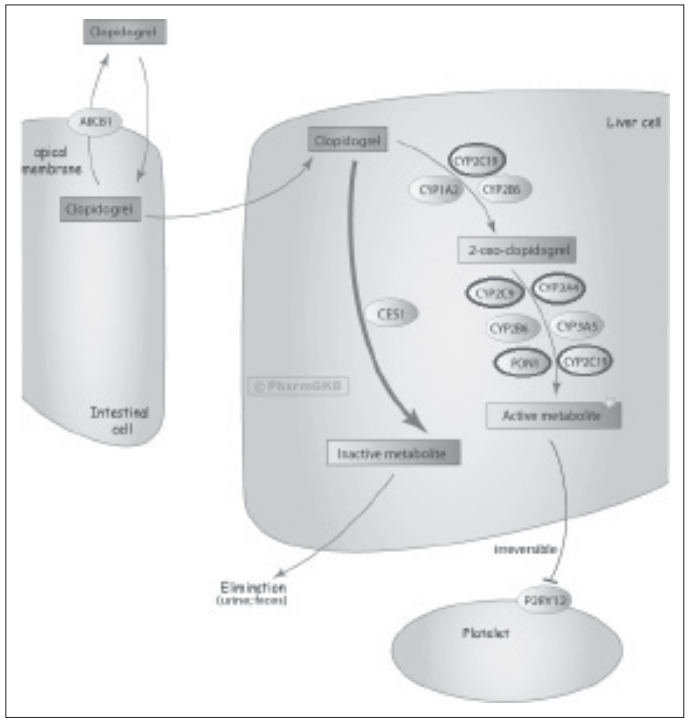

Figure 1. Hepatic metabolism of clopidogrel showing the enzymes involved. Those indicated with thick edges are coded by genes whose polymorphisms have been studied in this work. *With permission by PharmGKB, the Pharmacogenomics Knowledgebase (25)*

carried out in patients with ACS, and this is a special population with clinical and inflammatory peculiarities (16); although this study did not find a relationship between *CYP3A4*1B* and platelet reactivity, the statistical adjustment for clinical variables was relatively incomplete.

The most studied polymorphisms related to clopidogrel metabolism are found in CYP2C9, CYP2C19, and *PON1* genes (2, 17, 18). However, results of their influence on platelet reactivity have been contradictory, with *CYP2C19*2* being often associated with PRC (2, 18, 19).

Incomplete adjustment by confounders partly accounts for the different findings. For example, in a recent study (20) evaluating 25 polymorphisms, only a limited set of potential confounders [i.e., age, gender, cardiovascular risk factors, body mass index (BMI) and proton-pump inhibitors] was analyzed. However, it was concluded that *CYP2C19*2* allele tagged-SNP (single-nucleotide polymorphism) rs4244285 was a "strong" predictor of PRC. Further, no incremental value on prediction of PRC (above clinical variables) was provided by the authors. In this regard, a consensus is needed for statistical methods to properly assess the incremental value of a number of SNPs single polymorphisms or a genetic risk score in clinical practice (21). One set of metrics proposed for the assessment of novel markers in general, but not specifically for genetic markers, includes discrimination capacity (22). However, to date, only a limited number of prospective studies have assessed the incremental benefits (i.e., discrimination) of the genetic risk score over and abovementioned known clinical risk predictors (23).

Thus, in this study, we evaluated the contribution of clopidogrel-metabolizing enzyme polymorphisms on platelet reactivity in patients with ACS treated with clopidogrel over and above clinical and laboratory variables.

Methods

Population

We conducted an observational study, with cross-sectional analysis and prospective/consecutive data collection between June 2011 and January 2012. We included patients diagnosed with ACS, defined as typical chest pain and elevated markers of myocardial necrosis or T/ST-segment alterations suggestive of ischemia, remitted for cardiac catheterization and treated with clopidogrel ≥12 h, with a loading dose of 300 or 600 mg (physician choice).

In cases where clopidogrel loading dose could not be confirmed, patients were included if they were treated for at least 24 h after the first. Collected data for each patient encompassed baseline characteristics, including comorbidities and concomitant treatment.

Exclusion criteria were the presence of significant valvular heart disease or cardiomyopathy, concomitant diseases with life expectancy of <1 year, patients who did not sign the informed consent, and patients treated with platelet glycoprotein IIb/IIIa receptor antagonists. The study was approved by the Ethics Committee for Clinical Research at our center, and it complies with the Helsinki Declaration of 1975 and subsequent updates.

Platelet function

At the hemodynamic laboratory, we extracted 15 mL of peripheral blood from arterial sheath before using anticoagulants. We filled two tubes containing 3.2% sodium citrate (Vacuette®) and waited between 15 and 30 min before the evaluations, according to the manufacturer's instructions. The inhibitory effect of clopidogrel on platelet reactivity was measured with VerifyNow P2Y12® (Accumetrics Inc. San Diego, CA, USA). The instrument measures the change in light transmittance and the results were expressed as "Base PRU (Platelet Reactivity Units)": an estimate of the patient's baseline platelet function independent of P2Y12 receptor inhibition, "PRU": the amount of P2Y12 receptor-mediated aggregation, and "Percent inhibition [(PRU – Base PRU)/Base PRU × 100]": the difference between before and after clopidogrel treatment platelet reactivity. We used the cut-off level PRU=208 specified by the manufacturer as the definition of poor responders (24).

Genotyping

Peripheral blood samples were obtained from arterial sheath in EDTA tubes and DNA was extracted using the QIAamp® DNA minikit and automatic nucleic acid extractor QiaCube® (Qiagen, Hilden, Germany). Six SNPs tagging alleles involved in the me-

tabolism of clopidogrel were studied: *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285), *CYP2C19*17* (rs12248560), *CYP3A4*1B* (rs27405749), and *PON1*-Q192R (rs662) (25). Genotyping were determined by allelic discrimination using the TaqMan® Drug Metabolizing and the reactive GTXpress Master Mix (*CYP2C19*2*, *CYP2C19*17* and *PON1*-Q192R), provided by Applied Biosystems (Foster City, CA, USA), or KASPar® (*CYP2C9*2*, *CYP2C9*3* and *CYP3A4*1B*) based on FRET technology (Kbiosciences, Hertfordshire, UK).

Statistical analysis

Univariate analysis was performed using the chi-square test or Fisher's test for categorical variables and the Student's t-test for continuous variables to identify factors associated with PRC. PRC (PRU >208) was the dependent variable in the models of binary logistic regression. Covariates were those that showed association in the univariate analysis ($p<0.05$) or in previous studies under an explanatory perspective (age, gender, BMI, current smoking, type-2 diabetes mellitus, heart failure, acute myocardial infarction, estimated glomerular filtration rate, hemoglobin levels, concomitant statins and calcium-channel blockers). Covariates were entered in blocks using the backward stepwise method, applying the Wald statistic. Polymorphisms were introduced in a second block using the "enter" option. Odds ratios (OR) were calculated with their respective 95% confidence intervals (95% CI). The discrimination of the final model with and without the genetic score (six polymorphisms) was estimated using the C statistic, and the calibration using the Hosmer–Lemeshow test. Chi-squared score of each variable was estimated in the model as a method to assess the relative importance of each variable in the model. The first-degree interaction in the hierarchical model between variables loading dose and polymorphisms independently associated with PRC was analyzed. In addition, the C statistic for the model that only included clinical variables was compared by a hypothesis contrast test. A p value of <0.05 was considered statistically significant and all analyses were performed using the SPSS statistical package, version 20.0 (IBM, USA).

Results

Study population

We included 278 patients with a mean age of 66 years (standard deviation 11 years), and 85 (30.6%) were women. The baseline characteristics of the study sample are shown in Table 1. The diagnosis was ACS without persistent ST-segment elevation in 182 patients (65.5%), whereas it was ACS with persistent ST-segment elevation in 56 (20.1%). Poor responders had a significantly higher age; had a higher incidence of heart failure (Killip class >I); and had lower hematocrit, hemoglobin, and estimated glomerular filtration values were lower.

Troponin I elevation above the laboratory reference value was observed in 177 cases (63.7%). In 159 patients (57.2%), clopidogrel loading dose was administered, with 300 mg being the most common dose (n=139, 87.4%). The mean time from the first dose of clopidogrel until the determination of platelet aggregation was almost 9 days and the median was 5 days (interquartile range 2–10). At study enrolment, 231 (83.1%) were clopidogrel-naïve patients (Table 2).

Concomitant medications are also listed in Table 2. Of note, in 105 cases (37.8%), the patient received a proton-pump inhibitor, with pantoprazole being the most common (n=67, 63.8%); the most frequently used dose was 40 mg every 24 h (n=42, 62.7%). In 231 patients (83.1%), statins were administered, with a predominance of atorvastatin (n=215, 93.1%) 80 mg every 24 h (n=117, 54.4%). There were no significant differences in the use of concomitant medications during hospitalization between patients with and without PRC.

The angiographic characteristics are presented in Table 2. The mean number of vessels with significant lesions was 1.5±1.1 and the mean number of coronary lesions treated was 1.1±0.9 with 1.2±1.0 stents per person.

Patient profile with high on-treatment platelet reactivity

Overall PRU mean value was 261±78. According to response to clopidogrel, PRU was 164±35 in patients with adequate response and 295±58 in poor responders.

We identified 206 (74.1%) poor responders. In univariate analysis with platelet response (PRU>208 U) as the dichotomizing variable, we found that age (OR 1.63 per standard deviation, 95% CI 1.24–2.15), heart failure (OR 4.83, 95% CI 1.12–20.95), and the presence of ≥1 CYP2C19*2 allele (OR 2.01, 95% CI 1.03–3.93) were risk factors for high platelet reactivity. Current smoking (OR 0.41, 95% CI 0.23–0.72), hemoglobin (OR 0.65, 95% CI 0.54–0.77), hematocrit (OR 0.86, 95% CI 0.80–0.92), and estimated glomerular filtration rate (mL/min/1.73 m^2, evaluated with the Modification of Diet in Renal Disease formula OR 0.99, 95% CI 0.98–1.00) were found as protective factors. In a multiple logistic regression model that included age, sex, BMI, diabetes mellitus, current smoking, heart failure, acute myocardial infarction, baseline hemoglobin, estimated glomerular filtration rate, concomitant medication (calcium-channel blockers and statins), and the six polymorphisms (Table 3), the following were independent predictors of PRC risk: age (OR 1.43 per standard deviation, 95% CI 1.03–2.00), BMI (OR 4.03 per standard deviation, 95% CI 1.21–13.43), and the presence of ≥1 *CYP3A4*1B* allele (OR 4.05, 95% CI 1.01–16.34); on the other hand, high baseline hemoglobin (OR 1xe–18, 95% CI 1xe–26–1xe–10) was a protective factor. Female gender showed a tendency for being a protective factor (OR 0.44, 95% CI 0.20–1). The p value of Hosmer–Lemeshow test was 0.833. Chi-square score evaluation reported that (in order of importance) the most important variable in the model was hemoglobin, followed by age, *CYP2C9*2*, CYP2C19*2, and *CYP3A4*1B*.

Polymorphisms. Frequency and impact on platelet reactivity

The frequencies of *CYP3A4*1B*, *CYP2C9*2*, *CYP2C9*3*, CYP2C19*2, *CYP2C19*17*, and *PON1*-Q192R polymorphisms are

Table 1. Baseline characteristics

	Total cohort (n=278)	Poor responders (n=206, 74.1%)	Normal responders (n=72, 25.9%)	*P*
Age, years	65.9±11.2	67.3±11.0	61.9±11.0	<0.001
Female gender	85 (30.6)	66 (32.0)	19 (26.4)	0.370
BMI, kg/m^2	30.0±15.8	30.6±18.2	28.2±4.2	0.265
Risk factors				
Arterial hypertension	179 (64.4)	137 (66.5)	42 (58.3)	0.213
Dyslipidemia	162 (58.3)	121 (58.7)	41 (56.9)	0.791
Diabetes mellitus	108 (38.9)	83 (40.3)	25 (34.7)	0.404
Current smoking	86 (30.9)	53 (25.7)	33 (45.8)	0.001
Comorbidities				
FH ischemic heart disease	23 (8.3)	14 (6.8)	9 (12.5)	0.130
Ischemic heart disease	121 (43.5)	91 (44.2)	30 (41.7)	0.712
PPCA	84 (30.2)	61 (29.6)	23 (31.9)	0.711
Aortocoronary bypass	12 (4.3)	9 (4.4)	3 (4.2)	1
Stroke	10 (3.6)	8 (3.9)	2 (2.8)	1
Peripheral artery disease	11 (4.0)	7 (3.4)	4 (5.6)	0.483
COPD	18 (6.5)	16 (7.8)	2 (2.8)	0.172
CKD	19 (6.8)	17 (8.3)	2 (2.8)	0.173
Hospitalization				
Killip class > I	27 (9.7)	25 (12.1)	2 (2.8)	0.021
Changes in the ECG*	174 (62.6)	131 (63.6)	43 (59.7)	0.559
Troponin I elevation	177 (63.7)	126 (61.2)	51 (70.8)	0.142
Laboratory data				
Hemoglobin, g/dL	13.8±1.9	13.4±1.9	14.7±1.7	<0.001
Hematocrit, %	41.2±5.0	40.4±4.9	43.7±4.6	<0.001
Platelets, ×10^9/L	212.1±55.0	214.9±56.0	204.1±51.7	0.153
Leukocytes, –10^9/L	9.2± 8.4	9.6±9.5	8.2±2.9	0.228
Creatinine, mg/dL	1.0±0.5	1.0±0.5	0.9±0.3	0.092
MDRD, mL/min/1.73 m^2	84.0±28.8	81.4±26.5	91.3±33.7	0.013
Total cholesterol, mg/dL	176.0±48.5	172.1±47.0	186.2±51.1	0.045
HDL cholesterol, mg/dL	38.3±12.0	37.7±11.8	39.7±12.7	0.263
LDL cholesterol, mg/dL	107.3±39.2	105.6±39.2	111.8±39.1	0.294
Triglycerides, mg/dL	150.5±84.2	142.6±70.9	171.4±110.0	0.055

Quantitative variables are presented as mean±standard deviation and categorical variables as frequency and percentage. BMI - body mass index; CKD - chronic kidney disease; CODP - chronic obstructive pulmonary disease; ECG - electrocardiogram; FH - ischemic heart disease-Family history of ischemic heart disease; HDL - high-density lipoprotein; Killip class - presence of heart failure according to Killip and Kimball classification; LDL - low-density lipoproteins; MDRD - glomerular filtration rate according to the formula Modification of Diet in Renal Disease Brief; PPCA - Previous Percutaneous Coronary Angioplasty. *J point deviation ≥1 mm and/or presence of negative T-wave symmetry ≥3 mm except avR

shown in Table 4. Polymorphisms were in Hardy–Weinberg equilibrium (p=0.798, p=0.566, p=0.388, p=0.925, p=0.469, and p=0.210, respectively). The most common polymorphism in our study was the presence of at least one C allele of *PON1*-Q192R (55.0), whereas the most uncommon was the presence of at least one *1B allele of CYP3A4 (8.3%). The other polymorphisms showed frequencies between 16.2% and 38.1%. Poor responders presented a higher prevalence of CYP2C19 polymorphism and a tendency to significance was observed for *CYP2C9*2* polymorphism among normal responders. There were not differences between patients with and without PRC for the rest of polymorphisms.

Table 5 shows the aggregometry results associated with the six polymorphisms. The patients with at least one *CYP2C19*2* allele had significantly higher PRU values than patients with the original genotype, and the percent inhibition

Table 2. Angiographic data and hospital and discharge treatment

	Total cohort (n=278)	Poor responders (n=206, 74.1%)	Normal responders (n=72, 25.9%)	*P*
Concomitant treatment during hospitalization				
Salicylates	278 (100)	206 (100)	72 (100)	1
H2RAs	102 (36.7)	75 (36.4)	27 (37.5)	0.890
PPIs	105 (37.8)	80 (38.8)	25 (34.7)	0.517
ACE inhibitors or ARBs	206 (74.1)	149 (71.6)	57 (79.2)	0.225
Loop diuretics	51 (18.4)	43 (20.9)	8 (11.1)	0.061
Alpha-blockers	9 (3.2)	6 (2.9)	3 (4.2)	0.701
Beta-blockers	210 (75.5)	153 (74.3)	57 (79.2)	0.476
Nitrates	95 (34.2)	71 (34.5)	24 (33.3)	0.801
CCBs	45 (15.1)	36 (17.5)	9 (12.5)	0.295
Statins	231 (83.1)	168 (81.6)	63 (87.5)	0.309
Aldosterone antagonist	14 (5.0)	12 (5.8)	2 (2.8)	0.532
Clopidogrel				
Clopidogrel-naïve patients	231 (83.1%)	174 (84.5)	57 (79.2)	0.497
Time from the first dose, days	8.5±14.71	8.75±16.28	9.14±8.9	0.846
Loading dose	159 (57.2)	116 (56.3)	43 (59.7)	0.621
Loading dose of 300 mg	139 (87.4)	100 (86.2)	39 (90.7)	0.760
Cardiac catheterization				
Depressed LVEF	55 (19.8)	46 (22.3)	9 (12.5)	0.076
Number of diseased vessels	1.5±1.1	1.4±1.1	1.4±1.0	0.345
LMCA	16 (5.8)	12 (5.8)	4 (5.6)	1
LAD or its branches	153 (55.0)	115 (55.8)	38 (52.8)	0.823
Cx or its branches	106 (38.1)	76 (36.9)	30 (41.7)	0.375
RCA or its branches	137 (49.3)	109 (52.9)	28 (38.9)	0.062
Grafts	7 (2.5)	6 (2.9)	1 (1.4)	0.683
Treated lesions	1.1±0.9	1.1±0.8	1.2±1.1	0.594
Stents, units	1.2±1.0	1.2±1.0	1.3±1.3	0.442
≥1 Pharmacoactive stent	149 (53.6)	110 (53.4)	39 (54.2)	0.583
Total length, mm	29.2±18.2	29.0±17.2	29.9±21.1	0.765
Treatment at discharge				
Salicylates	250 (89.9)	184 (89.3)	66 (91.7)	0.461
Clopidogrel	213 (76.6)	156 (75.7)	57 (79.2)	0.437
Prasugrel	11 (4.0)	11 (5.3)	0 (0)	0.072
H2RAs	48 (17.3)	38 (18.5)	10 (13.9)	0.313
PPIs	100 (36.0)	75 (36.4)	25 (34.7)	0.637
ACE inhibitors or ARBs	207 (74.5)	152 (73.8)	55 (76.4)	0.946
Loop diuretics	48 (17.3)	41 (19.9)	7 (9.7)	0.040
Alpha-blockers	8 (2.9)	6 (2.9)	2 (2.8)	1
Beta-blockers	212 (76.3)	154 (74.8)	58 (80.6)	0.473
Nitrates	41 (14.8)	30 (14.6)	11 (75.3)	0.956
CCBs	44 (15.8)	35 (17.0)	9 (12.5)	0.319
Statins	231 (83.1)	171 (83.0)	60 (83.3)	0.553
Aldosterone antagonist	11 (4.0)	10 (4.9)	1 (1.4)	0.298
Acenocumarol	12 (4.3)	10 (4.9)	2 (2.8)	0.738

Quantitative variables are presented as mean±standard deviation and categorical variables as frequency and percentage. ACE - inhibitors-angiotensin-converting-enzyme inhibitors; ARBs - angiotensin II receptor blockers; CCBs - calcium-channel blockers; Cx - circumflex artery; H2RAs - Histamine-2 receptor antagonists; LAD - left anterior descending artery; LMCA - left main coronary artery; LVEF - left ventricular ejection fraction; PPIs - proton-pump inhibitors; RCA - right coronary artery

Table 3. Clinical-genetic model for prediction poor response to clopidogrel

	Adjusted OR[c]	95% CI	*P*	Chi[2]
Clinical variables				
Age, per each SD	1.43	1.03–2.00	0.034	11.28
Female gender	0.44	0.20–1.00	0.050	0.31
BMI, per each SD	4.03	1.21–13.43	0.024	1.43
Hemoglobin, g/dL[a]	$1xe^{-18}$	$1xe^{-26}$–$1xe^{-10}$	<0.001	23.17
Concomitant statins[b]	0.43	0.17–1.09	0.074	0.64
Genetic variables				
CYP3A4 ≥1 allele 1B	4.05	1.01–16.34	0.049	1.72
CYP2C9 ≥1 allele *2	0.62	0.32–1.23	0.170	3.94
CYP2C9 ≥1 allele *3	1.35	0.55–3.35	0.517	0.26
CYP2C19 ≥1 allele *17	1.73	0.83–3.59	0.145	1.61
CYP2C19 ≥1 allele *2	2.03	0.92–4.50	0.081	2.77
PON1 Q192R ≥1 allele C	0.54	0.18–1.67	0.287	1.54

[a]Variable transformed by the logarithm of decimal base; [b]91.3% were atorvastatin takers; [c]Adjusted for current smoking, diabetes, heart failure, acute myocardial infarction, estimated glomerular filtration rate and concomitant calcium-channel blockers. BMI - body mass index; CI - confidence interval; OR - odds ratio; SD - standard deviation. Model calibration: Hosmer–Lemeshow test: χ^2=4.256; df=8; *P* value=0.833

was also significantly lower in the former. Patients carrying *CYP3A4*1B* allele presented higher values of PRU and lower values of inhibition, although this did not reach statistical significance. The same was observed when the analysis was dichotomized by PRU of <208 U, i.e., more normal responders among wild-type carriers.

Discrimination of poor response to clopidogrel: clinical and genetic variables

The C statistic for the model presented above was 0.749 (95% CI 0.683–0.815) and for the model that included *CYP3A4*1B* was 0.763 (95% CI 0.699–0.826, p for area comparison=0.088).

Furthermore, the C statistic for the model that included clinical variables and the six polymorphisms was 0.788 (95% CI 0.729–0.847, p for area comparison versus clinical model=0.028) (Fig. 2). The increment of the discrimination capacity compared to the clinical model was 3.9%.

Discussion

The main result of our study suggests that the presence of at least one *CYP3A4*1B* allele can independently influence platelet response to clopidogrel in patients with ACS in an exhaustively adjusted model. The relative importance of *CYP3A4*1B* as a predictor of response to clopidogrel is modest compared with clinical or routine laboratory variables such as age, sex, BMI, and baseline hemoglobin, as suggested the analysis of discrimination and the chi-square score. Importantly, our analysis further shows that a clinical model built with five (age, gender, BMI, hemoglobin, and concomitant statins) easily obtained variables yielded a good discrimination in the identification of patients with poor response to clopidogrel, and even though the discrimination significantly improves when a set of six polymorphisms is added compared to the clinical model, this increase is poor.

There have been few studies that evaluate the effect of *CYP3A4*1B* on the response to clopidogrel (9–15). Of these, only one previous study (13) has evaluated the prognostic value of *CYP3A4*1B* in patients with ACS under similar conditions to ours. That study recruited 603 patients with ACS without ST-segment elevation and yielded a negative result (13); remarkably, the analysis for prediction of platelet reactivity was performed by adjusting it only for age and sex. Nevertheless, in our study, the model was carefully adjusted for age, sex, BMI, diabetes, current smoking, heart failure, acute myocardial infarction, baseline hemoglobin, estimated glomerular filtration rate, concomitant medication, and the six polymorphisms. These substantial differences in the statistical adjustment may have had a role in the discordance between the findings of the two studies. The remaining studies have been conducted in other patient types (9–12, 14, 15).

Some studies have shown that other CYP3A4 polymorphisms influence the response to clopidogrel (2, 10). However, the associations for polymorphism *CYP3A4*1B* could not be confirmed (9–12, 14, 15); this may be because of the small sample size of the studies (9, 10, 15) and the low prevalence of the polymorphism (9, 10), which did not allow for the analysis.

Moreover, Brandt et al. (15) performed a study in healthy subjects and Angiolillo et al. (10) in patients with stable coronary artery disease. Our results are not comparable to those found

Table 4. Proportion of polymorphisms related to hepatic metabolism of clopidogrel

	Total cohort (n=278)	Poor responders (n=206, 74.1%)	Normal responders (n=72, 25.9%)	*P*
CYP3A4 ≥1 allele 1B, n (%)	23 (8.3)	19 (9.2)	4 (5.6)	0.354
CYP2C9 ≥1 allele *2, n (%)	90 (32.4)	61 (29.6)	29 (40.3)	0.071
CYP2C9 ≥1 allele *3, n (%)	45 (16.2)	35 (17.0)	10 (13.9)	0.596
CYP2C19 ≥1 allele *17, n (%)	106 (38.1)	83 (40.3)	23 (31.9)	0.209
CYP2C19 ≥1 allele *2, n (%)	76 (27.3)	63 (30.6)	13 (18.1)	0.038
PON1-Q192R ≥1 allele C, n (%)	153 (55.0)	111 (53.9)	42 (58.3)	0.539

Qualitative variables are presented as frequency and percentage.

Table 5. Aggregometry results for polymorphisms related to hepatic metabolism of clopidogrel and platelet reactivity

CYP3A4	wt/wt (n=252, 90.7%)	wt/*1B (n=23, 8.3%)	*1B/*1B (n=0)	*P*
Base PRU	300.0±54.5	284.6±64.8	–	0.307
PRU	261.4±78.9	267.0±67.8	–	0.755
PRU <208	66 (26.2%)	4 (17.4%)	–	0.354
Percent inhibition	15.9±17.7	11.9±15.5	–	0.398
Reactivity time (RT)	5.0 (8)	7.0 (18)	–	0.311
CYP2C9 allele 2	wt/wt (n=177, 63.7%)	wt/*2 (n=80, 28.8%)	*2/*2 (n=10, 3.6%)	*P*
Base PRU	299.0±55.1	301.4±57.0	293.9±50.9	0.677
PRU	265.9±78.7	252.1±79.3	280.3±63.2	0.423
PRU <208	39 (22.0%)	28 (35.0%)	1 (10.0%)	0.045
Percent inhibition	14.7±17.2	18.0±18.7	12.6±14.3	0.247
Reactivity time (RT)	5.0 (9)	4.5 (8)	5.5 (4)	0.703
CYP2C9 allele 3	wt/wt (n=232, 83.5%)	wt/*3 (n=41, 14.8%)	*3/*3 (n=1, 0.3%)	*P*
Base PRU	299.1±55.1	291.5±50.7	400.0	0.219
PRU	262.8±79.1	257.3±73.5	288.0	0.789
PRU <208	59 (25.4%)	11 (26.8%)	0	0.827
Percent inhibition	15.6±17.8	14.4±16.1	28.0	0.613
Reactivity time (RT)	5.0 (8)	5.0 (6)	12.0	0.513
CYP2C19 allele 2	wt/wt (n=201, 72.3%)	wt/*2 (n=72, 25.9%)	*2/*2 (n=4, 1.4%)	*P*
Base PRU	298.9±54.2	299.0±59.6	326.5±5.3	0.391
PRU	251.9±76.1	285.3±81.3	298.8±33.4	0.020
PRU <208	50 (24.9%)	13 (18.1%)	0	0.084
Percent inhibition	17.7±18.4	10.8±14.9	6.5±9.0	0.006
Reactivity time (RT)	5.0 (9)	6.0 (7)	6.5 (6)	0.416
CYP2C19 allele 17	wt/wt (n=172, 61.9%)	wt/*17 (n=93, 33.4%)	*17/*17 (n=13, 4.7%)	*P*
Base PRU	297.2±57.1	299.9±53.3	312.5±52.1	0.660
PRU	261.7±81.8	259.3±71.5	166.5±83.2	0.882
PRU <208	49 (28.5%)	22 (23.7%)	1 (7.7%)	0.213
Percent inhibition	16.0±17.5	15.2±17.8	18.1±21.3	0.848
Reactivity time (RT)	6.0 (8)	4.0 (8)	5.0 (9)	0.157
PON1 rs662	TT (n=121, 43.5%)	CT (n=115, 41.4%)	CC (n=38, 13.7%)	*P*
Base PRU	298.0±55.8	299.8±56.0	300.3±51.9	0.961
PRU	263.1±75.4	254.3±79.5	275.6±84.1	0.482
PRU <208	30 (24.8%)	36 (31.3%)	6 (15.8%)	0.139
Percent inhibition	15.2±17.3	17.2±18.5	14.1±17.1	0.479
Reactivity time (RT)	4.0 (8)	5.0 (7)	7.0 (6)	0.217

Quantitative variables are presented as mean±standard deviation or median (IR) and categorical variables as frequency and percentage. PRU - platelet reactivity units; RT - reactivity time between first dose of clopidogrel and determination of reactivity, median of days (interquartile range); wt - wild type

in patients undergoing "elective" coronary intervention (9, 12), those by Geisler et al. (14) found in a mixed population in which only 45.5% had ACS, or those of Harmsze et al. (11) found in study with a different design—cases and controls—in patients with a history of stent thrombosis. Our study, on the other hand, was conducted in a population of ACS patients referred for cardiac catheterization.

Several studies have associated the *CYP2C19*2* polymorphism with higher values of PRU and an increase of cardiovascular adverse effects (17, 18, 26). However, in our analysis, it was not an independent predictor or PRC, although it presented the second highest chi-square score of genetic variables. Our results are in contrast with those of Park et al. (2), which examined the relationship between a panel of clinical variables and nine

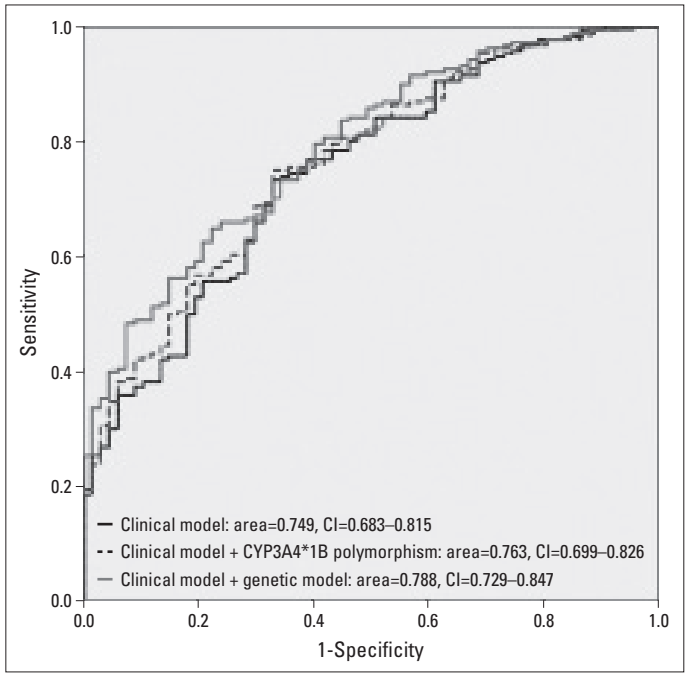

Figure 2. Receiver operating characteristic curves for the clinical model, the model with clinical variables and CYP3A4*1B and the model with clinical variables and the six polymorphisms

polymorphisms—not including *CYP3A4*1B*—with high platelet reactivity. The only genetic variable resulting as independent predictor was *CYP2C19*2*. The other polymorphisms did not affect platelet reactivity. Race-specific differences and a smaller sample size might be factors accounting for such differences between Park et al.'s (2) study and ours.

In our patient cohort, we observed a high prevalence of PRC, although in line with previous reports (27, 28). Thus, Tousek et al. (27) found a prevalence of 80% PRC in patients with severe aortic stenosis who underwent implantation of transcatheter aortic valve replacement and were treated with clopidogrel, whereas Kang et al. (28) reported a prevalence of 69.8% in patients undergoing elective percutaneous coronary intervention. This fact may be related to the inclusion of patients with ST elevation ACS–who are exposed to clopidogrel for shorter time—and the decision to choose the PRU cut-off level of 208 units, clearly lower than that used in previous studies (2, 17), although our value is based on the recent manufacturer recommendations (24). In this regard, authors have shown considerable controversy not only about the method to quantify the platelet reactivity but also the optimal cut-off to define PRC (29, 30). Another factor that may have influence was the adoption of the inclusion criteria based on treatment duration with clopidogrel of ≥12 h after the loading dose. This decision was based on previous pharmacokinetic studies indicating that the equilibrium state ("steady state") of clopidogrel could be achieved at 5 h from the initial loading dose (31). Moreover, the optimal cut off value might be different in the acute phase of an ACS in comparison with other scenarios, such as stable coronary disease or elective percutaneous coronary intervention.

The relationship between diabetes mellitus and PRC is well known and has been previously documented (32–34) with a few exceptions (28, 35). In this line, Kang et al. (28) did not find that the presence of diabetes mellitus was an independent predictor of high platelet reactivity (OR=1.681, 95% CI 0.750–3.759). In our study, we did not observe an association between diabetes mellitus and PRC. We do not have an explanation for such a puzzling finding, but we speculate that the relatively small sample of our study and the one by Kang (28) and Park (35) might have played a role. In our study, patients with high platelet reactivity were older, had higher BMI, and had anemia, which is also in line with previous studies (2, 36, 37).

Although there is limited experience, investigators are evaluating a potential usefulness of the determination of certain polymorphisms by point-of-care systems to facilitate decisions regarding the optimal antiplatelet therapy in the initial phase of the ACS patient care. This might represent also a clinical scenario where the determination of these polymorphisms might help to take decisions in short periods of time. In this regard, pilot studies suggest that these determinations may help to identify patients who benefit from a second loading dose of clopidogrel or a more potent antiplatelet drug (38, 39).

Future studies will clarify whether genotyping may help to decide the optimal treatment in patients with ACS. Probably, as shown by our results, the future is not in the analysis of a single polymorphism but a panel of them, among which *CYP3A4*1B* could be taken into account. Finally, in the era of "supermachines," with the development of technologically advanced and expensive techniques, information obtained from medical notes and simple blood tests might still be useful in the identification of a significant proportion of patients with poor response to clopidogrel.

Study limitations

Our study has a few limitation. First, the sample size was modest and was determined by the maximum number of patients recruited in the interval indicated. In second place, although unlikely, the low prevalence of the *CYP3A4*1B* allele may have had an impact on the findings. However, allelic prevalence was similar to other studies (13, 15) and it was in accordance with the Hardy–Weinberg equilibrium (13) and the number of variables in the adjusted model had a relationship greater than 10 respect to the dependent variable. Another potential limitation is the determination of platelet reactivity at only one time point. As strength, we note that is a study, which evaluates six SNPs as well as a set of clinical variables in a population of patients with ACS.

Conclusion

*CYP3A4*1B* polymorphism may be an independent determinant of poorer response to clopidogrel in patients with ACS, although the variability in response to clopidogrel explained by the six polymorphisms is poor when compared with clinical variables.

Financial disclosure: The present study was funded by a grant "Investigadores noveles FFIS/CM10/006".

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – M.H.G.L., L.C.S., P.C.Z.; Design – M.H.G.L., L.C.S., P.C.Z.; Supervision –; Fundings –; Materials –; Data collection &/or processing – L.C.S., P.C.V.; Analysis &/or interpretation – M.H.G.L., L.C.S., P.C.Z., J.R.C., F.S.A., L.G.D.G., P.C.V., J.A.C.M., A.M.M.; Literature search – M.H.G.L., L.C.S., P.C.Z., A.M.M., J.A.C.M.; Writing – N.Ç.; Critical review – M.H.G.L., L.C.S., P.C.Z., A.M.M., J.A.C.M.

References

1. Jin HY, Yang TH, Kim DI, Chung SR, Seo JS, Jang JS, et al. High post-clopidogrel platelet reactivity assessed by a point-of-care assay predicts long-term clinical outcomes in patients with ST-segment elevation myocardial infarction who underwent primary coronary stenting. Int J Cardiol 2013; 167: 1877-81. [CrossRef]
2. Park JJ, Park KW, Kang J, Jeon KH, Kang SH, Ahn HS, et al. Genetic determinants of clopidogrel responsiveness in Koreans treated with drug-eluting stents. Int J Cardiol 2013: 163: 79-86. [CrossRef]
3. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 2009; 360: 354-62. [CrossRef]
4. Ko YG, Suh JW, Kim BH, Lee CJ, Kim JS, Choi D, et al. Comparison of 2 point-of-care platelet function tests, VerifyNow Assay and Multiple Electrode Platelet Aggregometry, for predicting early clinical outcomes in patients undergoing percutaneous coronary intervention. Am Heart J 2011; 161: 383-90. [CrossRef]
5. Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB, et al. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 2009; 302: 849-57. [CrossRef]
6. Angiolillo DJ, Alfonso F. Platelet function testing and cardiovascular outcomes: steps forward in identifying the best predictive measure. Thromb Haemost 2007; 98: 707-9. [CrossRef]
7. Angiolillo DJ, Bernardo E, Sabate M, Jimenez-Quevedo P, Costa MA, Palazuelos J, et al. Impact of platelet reactivity on cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2007; 50: 1541-7. [CrossRef]
8. Huber K. Genetic variability in response to clopidogrel therapy: clinical implications. Eur Heart J 2010; 31: 2974-6. [CrossRef]
9. Gladding P, Webster M, Zeng I, Farrell H, Stewart J, Ruygrok P, et al. The pharmacogenetics and pharmacodynamics of clopidogrel response: an analysis from the PRINC (Plavix Response in Coronary Intervention) trial. JACC Cardiovasc Interv 2008; 1: 620-7. [CrossRef]
10. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramírez C, Cavallari U, Trabetti E, et al. Contribution of gene sequence variations of the hepatic cytochrome P450 3A4 enzyme to variability in individual responsiveness to clopidogrel. Arterioscler Thromb Vasc Biol 2006; 26: 1895-900. [CrossRef]
11. Harmsze AM, van Werkum JW, Ten Berg JM, Zwart B, Bouman HJ, Breet NJ, et al. CYP2C19*2 and CYP2C9*3 alleles are associated with stent thrombosis: a case-control study. Eur Heart J 2010; 31: 3046-53. [CrossRef]
12. Harmsze A, van Werkum JW, Bouman HJ, Ruven HJ, Breet NJ, Ten Berg JM, et al. Besides CYP2C19*2, the variant allele CYP2C9*3 is associated with higher on-clopidogrel platelet reactivity in patients on dual antiplatelet therapy undergoing elective coronary stent implantation. Pharmacogenet Genomics 2010; 20: 18-25. [CrossRef]
13. Frere C, Cuisset T, Morange PE, Quilici J, Camoin-Jau L, Saut N, et al. Effect of cytochrome p450 polymorphisms on platelet reactivity after treatment with clopidogrel in acute coronary syndrome. Am J Cardiol 2008; 101: 1088-93. [CrossRef]
14. Geisler T, Schaeffeler E, Dippon J, Winter S, Buse V, Bischofs C, et al. CYP2C19 and nongenetic factors predict poor responsiveness to clopidogrel loading dose after coronary stent implantation. Pharmacogenomics 2008; 9: 1251-9. [CrossRef]
15. Brandt JT, Close SL, Iturria SJ, Payne CD, Farid NA, Ernest II CS, et al. Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. J Thromb Haemost 2007; 5: 2429-36. [CrossRef]
16. Gori AM, Cesari F, Marcucci R, Giusti B, Paniccia R, Antonucci E, et al. The balance between pro- and anti-inflammatory cytokines is associated with platelet aggregability in acute coronary syndrome patients. Atherosclerosis 2009; 202: 255-62. [CrossRef]
17. Viviani Anselmi C, Briguori C, Roncarati R, Papa L, Visconti G, Focaccio A, et al. 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. JACC Cardiovasc Interv 2013; 6: 1166-75.
18. Liang ZY, Han YL, Zhang XL, Li Y, Yan CH, Kang J. The impact of gene polymorphism and high on-treatment platelet reactivity on clinical follow-up: outcomes in patients with acute coronary syndrome after drug-eluting stent implantation. EuroIntervention 2013; 9: 316-27. [CrossRef]
19. Tello-Montoliu A, Jover E, Marin F, Bernal A, Lozano ML, Sánchez-Vega B, et al. Influence of CYP2C19 polymorphisms in platelet reactivity and prognosis in an unselected population of non ST elevation acute coronary syndrome. Rev Esp Cardiol 2012; 65: 219-26.
20. Cui H, Lin S, Chen X, Gao W, Li X, Zhou H, et al. Correlation between SNPs in candidate genes and VerifyNow- detected platelet responsiveness to aspirin and clopidogrel treatment. Cardiovasc Drugs Ther 2015; 29: 137-46. [CrossRef]
21. Fox CS, Hall JL, Arnett DK, Ashley EA, Delles C, Engler MB, et al. Future translational applications from the contemporary genomics era: a scientific statement from the American Heart Association. Circulation 2015; 131: 1715-36. [CrossRef]
22. Hlatky MA, Greenland P, Arnet DK, Ballantine CM, Criqui MH, Elkind MS, et al. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. Circulation 2009; 119: 2408-16. [CrossRef]
23. Ripatti S, Tikkanen E, Orho-Melander M, Havulinna AS, Silander K, Sharma A, et al. A multilocus genetic risk score for coronary heart disease: case-control and prospective cohort analyses. Lancet 2010; 376: 1393-400. [CrossRef]
24. VerifyNow P2Y12 instructions (2013) Analysis of platelet reactivity. Accumetrics. http://www.accriva.com/products/verifynow-system-platelet-reactivity-test. Accessed 1 Apr 2016.
25. The Pharmacogenomics Knowledgebase. http://www.pharmgkb.org. Accessed 15 Apr 2016.
26. Sibbing D, Gebhard D, Koch W, Braun S, Stegherr J, Morath T, et al. Isolated and interactive impact of common CYP2C19 genetic variants on the antiplatelet effect of chronic clopidogrel therapy. J Thromb Haemost 2010; 8: 1685-93. [CrossRef]
27. Tousek P, Kocka V, Sulzenko J, Bednar F, Linkova H, Widimsky P. Pharmacodynamic effect of clopidogrel in patients undergoing transcatheter aortic valve implantation. Biomed Res Int 2013: 386074.

28. Kang MK, Jeong YH, Yoon SE, Koh JS, Kim IS, Park Y, et al. Pre-procedural platelet reactivity after clopidogrel loading in korean patients undergoing scheduled percutaneous coronary intervention. J Atheroscler Thromb 2010; 17: 1122-31. [CrossRef]
29. Bonello L, Tantry US, Marcucci R, Blindt R, Angiolillo DJ, Becker R, et al. Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol 2010; 56: 919-33. [CrossRef]
30. Consuegra-Sánchez L, López-Palop R, Cano P, Carrillo P, Picó F, Villegas M, et al. Assessment of high on-treatment platelet reactivity in patients with ischemic heart disease: concordance between the Multiplate and VerifyNow assays. J Thromb Haemost 2013; 11: 379-81
31. Savcic M, Hauert J, Bachmann F, Wyld PJ, Geudelin B, Cariou R. Clopidogrel loading dose regimens: kinetic profile of pharmacodynamic response in healthy subjects. Semin Thromb Hemost 1999; 25: 15-9.
32. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramírez C, Sabate M, Jimenez-Quevedo P, et al. Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. Diabetes 2005; 54: 2430-5. [CrossRef]
33. Serebruany V, Pokov I, Kuliczkowski W, Chesebro J, Badimon J. Baseline platelet activity and response after clopidogrel in 257 diabetics among 822 patients with coronary artery disease. Thromb Haemost 2008; 100: 7682. [CrossRef]
34. Ferreiro JL, Angiolillo DJ. Diabetes and antiplatelet therapy in acute coronary syndrome. Circulation 2011; 123: 798-813. [CrossRef]
35. Park KJ, Chung HS, Kim SR, Kim HJ, Han JY, Lee SY. Clinical, pharmacokinetic, and pharmacogenetic determinants of clopidogrel resistance in Korean patients with acute coronary syndrome. Korean J Lab Med 2011; 31: 91-4. [CrossRef]
36. Bouman HJ, Harmsze AM, van Werkum JW, Breet NJ, Bergmeijer TO, Ten Cate H, et al. Variability in on-treatment platelet reactivity explained by CYP2C19*2 genotype is modest in clopidogrel pretreated patients undergoing coronary stenting. Heart 2011; 97: 1239-44. [CrossRef]
37. Toma C, Zahr F, Moguilanski D, Grate S, Semaan RW, Lemieux N, et al. Impact of anemia on platelet response to clopidogrel in patients undergoing percutaneous coronary stenting. Am J Cardiol 2012; 109: 1148-53. [CrossRef]
38. Lee JA, Lee CR, Reed BN, Plitt DC, Polasek MJ, Howell LA, et al. Implementation and evaluation of a CYP2C19 genotype-guided antiplatelet therapy algorithm in high-risk coronary artery disease patients. Pharmacogenomics 2015; 16: 303-13. [CrossRef]
39. Stimpfle F, Karathanos A, Droppa M, Metzger J, Rath D, Müller K, et al. Impact of point-of-care testing for CYP2C19 on platelet inhibition in patients with acute coronary syndrome and early dual antiplatelet therapy in the emergency setting. Thromb Res 2014; 134: 105-10. [CrossRef]

From Prof. Dr. Arif Akşit's collections