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The *CYP2C19*2* and *CYP2C19*17* Polymorphisms play a Vital Role in Clopidogrel Responsiveness after Percutaneous Coronary Intervention: A Pharmacogenomics Study

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Abstract: Clopidogrel inhibits platelet activation and aggregation by blocking the $P2Y_{12}$ receptor. Dual antiplatelet therapy with clopidogrel and aspirin is recommended treatment by current guidelines for patients undergoing percutaneous interventions. Recurrent ischaemic cardiac events after this treatment showed lack of clopidogrel responsiveness. We aimed to investigate the most noticeable variants in the genes involved in clopidogrel pharmacokinetics and pharmacodynamics. A total of 347 Turkish patients who underwent percutaneous coronary interventions with stent implantation were included in our study. Platelet reactivity (PRU) and % inhibition were measured with VerifyNow $P2Y_{12}$ assay in blood samples collected from patients who took a standard dose of clopidogrel (75 mg/day) for at least 7 days. The variants in the *CYP2C19*, *CYP3A4*, *CYP2B6*, *ABCB1*, *ITGB3* and *PON1* genes were genotyped using the Sequenom MassARRAY system. When grouped, the patients with PRU values >208 as non-responsiveness to clopidogrel therapy; 104 (30%) patients were non-responders and 243 (70%) patients were responders. A significant association was found between the *CYP2C19*2* (G636A) polymorphism and non-responsiveness to clopidogrel therapy ($p < 0.001$). An allele frequency of this single nucleotide polymorphism was high in non-responders; its odds ratio was 2.92 compared with G allele ($p < 0.001$). PRU values of CT genotypes were lower ($p = 0.029$) and % inhibition values of CT genotypes were higher ($p = 0.008$) compared with CC genotypes for the *CYP2C19*17* (C806T) polymorphism. None of the other genetic variants were found to be statistically associated with non-responsiveness to clopidogrel and antiplatelet activity. Our findings suggest that the *CYP2C19*2* polymorphism is associated with non-responsiveness to clopidogrel therapy and the *CYP2C19*17* polymorphism enhances antiplatelet activity of clopidogrel. Depending on haplotypes of these two polymorphisms, clopidogrel-treated patients can be protected or not from stent thrombosis and ischaemic events.

Dual antiplatelet medication with aspirin and a thienopyridine agent is currently recommended strategy for patients with coronary artery disease (CAD) undergoing percutaneous coronary intervention (PCI). Current guidelines suggest that this treatment continues for at least 1 year after stent implantation [1]. Today, the most widely prescribed thienopyridine is clopidogrel. It is the second best-selling drug with over $9 bn a year in global sales [2]. However, dual medication is even used, and recurrent atherothrombotic events often resulting in fatal stent thrombosis are a major drawback. It is shown that these undesirable cardiovascular results are due to low clopidogrel responsiveness in some patients. This phenomenon has been called 'clopidogrel resistance' by many scientists [3–5]. This resistance is seen in 25% of clopidogrel-treated patients on average [6]. The mechanisms underlying the response variability are not clear and still subject to discussion. According to a genomewide association study, 83% of interindividual variability in response to clopidogrel is caused by genetic polymorphisms [7].

Clopidogrel, an irreversible inhibitor of the $P2Y_{12}$ receptor on platelet membrane, is an oral pro-drug that requires metabolic transformation. After intestinal absorption by an efflux pump coded by the *ABCB1* gene, approximately 90% of clopidogrel is hydrolysed to inactive metabolites by carboxylesterase 1 (CES1). The minority is converted to active metabolites by the cytochrome P450 (CYP) enzymes in a two-step process. While CYP2C19 carries the most active role, CYP2B6, CYP1A2, CYP3A4/5 and CYP2C9 have less active contribution in this hepatic conversion [6]. Active metabolites of clopidogrel then irreversibly bind the adenosine diphosphate receptor (ADP) coded by the *$P2Y_{12}$* gene and inhibit activation of the glycoprotein IIb/IIIa complex coded by the *ITGB3* gene involved in platelet aggregation [1,8]. Previous studies have pointed to the variants in the genes encoding proteins involved in this pathway of the clopidogrel bioactivation; especially, the CYP enzymes have attracted attention because of their polymorphic character. CYP2C19

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polymorphism is the major determinant in platelet reactivity in response to clopidogrel. This finding was first published by Kim *et al.* [9] and was further refined by Holmes *et al.* [10]. The loss of function alleles (*2, *3, *4, *5, *6, *7 and *8) associated with an increased risk of ischaemic events and stent thrombosis have been detected on the *CYP2C19* gene by genetic and clinical studies [6,11]. In contrast, it is shown that a different allele (*17) on the same gene is a gain-of-function variant associated with an increased activation of clopidogrel [6]. As a result of accelerated molecular studies on the variability of clopidogrel response, scientists have suggested a new protein involved in the metabolism of clopidogrel as the rate-limiting enzyme. This protein initially propounded by Bouman *et al.* [12] is paraoxonase-1 (PON1), and its common Q192R (rs662) polymorphism was found associated with increased risk of stent thrombosis. However, the following other studies have reported that there is no such association [6].

The variability of response to clopidogrel has been studied mostly in Western populations until today, however with conflicting findings, and its boundaries have not been fully drawn. This variability based on mostly genetic variants can be illuminated more clearly in cohorts from different origins. We therefore aimed to analyse the variants on the genes encoding proteins included in the pharmacokinetic and pharmacodynamic response to clopidogrel in a different cohort. The genotypes of these variants were first assessed with antiplatelet activity in a Turkish population as a pharmacogenomics study.

Materials and Methods

Subjects and study design. Eskisehir Osmangazi University Clinical Trials Ethics Committee approved all the protocols of the study. A total of 1180 patients were admitted to Eskisehir Osmangazi University Medical Faculty, Department of Cardiology, for percutaneous coronary intervention with elective or emergent stent implantation between 1 September 2013 and 1 April 2015. Patients with a variety of coronary syndromes, including non-ST-segment elevation acute coronary syndromes (ACS) were enrolled. Percutaneous coronary intervention was performed according to the current standard guidelines. A total of 473 patients, who had a history of bleeding disorders, chronic liver disease, severe chronic renal failure, up to 80 years of age, used antiplatelet agents other than clopidogrel, anticoagulant or omeprazole as a proton pump inhibitor, whose platelet count was lower than 150 000/mm^3, haematocrit value was <33% or serum creatinine value was >2.5 mg/dl, were excluded from the study. The inclusion criterion for our study was use of clopidogrel 75 mg/day and aspirin 150–300 mg/day continuously for at least 7 days. A total of 345 patients who did not receive clopidogrel and aspirin at these maintenance doses were not included. In addition, 15 patients were excluded due to haemolysis of the blood samples collected for the platelet function test. As a result, 347 of 1180 Turkish patients, who did not have any familial relations to one another, were included in our study. These patients were informed about the content of the study and written consent form was received from all of them.

We selected the *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893), *CYP2C19*4* (rs28399504), *CYP2C19*7* (rs72558186), *CYP2C19*8* (rs41291556) and *CYP2C19*17* (rs12248560) variants according to the US Food and Drug Administration (FDA) box warning on 12 March 2012 [11]. This declaration was a result of the pharmacogenetics studies conducted until that time in Western countries. The *ABCB1* C3435T (rs1045642), *CYP3A4*1G* (rs2242480), *CYP2B6*9* (rs3745274), *ITGB3* L33P (rs5918), *PON1* Q192R (rs662) and *PON1* L55M (rs854560) variants were selected from the articles that found the most significant relationship [12–16]. All of these variants were first evaluated together with pharmacodynamic findings in the Turkish population.

DNA isolation and genotyping. DNA was isolated from 200 μL of peripheral blood anticoagulated with ethylenediaminetetraacetic acid using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The amount and purity of the DNA samples were optimized using a Thermo Scientific NanoDrop™ 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) spectrophotometer. Then, primers for the amplification and extension PCRs were designed in the Sequenom Assay Designer 3.1 (Sequenom, San Diego, CA, USA) software. These primer sequences shown in the supplementary material (Appendix S1) were supplied from the manufacturer. The variants were then genotyped by single-base extension reactions (iPLEX, Sequenom, San Diego, CA, USA) using the MassARRAY system (Sequenom, San Diego, CA, USA). This system uses MALDI-TOF mass spectrometry to analyse the amplicons. The MassARRAY system with high levels of accuracy is widely used for fine mapping and validation of GWASs.

Pharmacodynamic assessment of platelet reactivity. For the measurement of platelet reactivity, peripheral blood samples were obtained by venipuncture after a 5-mL discard sample. Each sample was collected into 1.8-mL blood collection tubes containing 3.2% citrate. Platelet reactivity was then measured using the VerifyNow $P2Y_{12}$ assay (Accumetrics, San Diego, CA, USA) according to the manufacturer's instructions. This assay containing fibrinogen-coated beads measures adenosine diphosphate-induced aggregation as an increase in light and reports the reactivity as $P2Y_{12}$ reaction units (PRUs). Nonetheless, the level of platelet inhibition is calculated as a percentage (% inhibition) by the system. According to the consensus on the definition of on-treatment platelet reactivity, PRU>208 is associated with post-PCI ischaemic events and increased risk of cardiovascular death [17]. Thus, the cut-off of 208 PRU was used to assess responder and non-responder status.

Statistical analysis. Statistical evaluation of the data was performed with IBM SPSS (Statistical Package for Social Sciences) for Windows 20.0. Kolmogorov–Smirnov and Shapiro–Wilk tests were used for the compliance of the data with the normal distribution. Of the parametric tests, independent-samples *t*-test and one-way ANOVA test were used for the analysis of data showing normal distribution in the comparison between the groups. Of the post hoc tests, Tukey's test was used for determining the averages of the different groups. Parametric data showing normal distribution was shown as mean ± SD (standard deviation). Of the nonparametric tests, the Mann–Whitney *U*-test and Kruskal–Wallis test were used for the analysis of data without normal distribution in the comparisons between the groups. Analysis was made using Dunn's test for the determination of different groups. Findings not showing normal distribution were shown as 25th percentile–75th percentile with median. Chi-square analyses (Pearson, Pearson exact, Yates and Fisher's exact tests) were used for the analysis of cross-tabs. Stepwise logistic regression analysis was used for determining the risk of genotype and allele of the *CYP2C19*2* (rs4244285) polymorphism, which is thought to be effective in responding and not responding to clopidogrel treatment. $p < 0.05$ was accepted as statistically significant.

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Results

Clopidogrel responsiveness and characteristics of the patients. Platelet reactivity and inhibition values (%) of 347 patients were measured by the VerifyNow $P2Y_{12}$ assay. A total of 104 (30%) patients whose PRU value was more than 208 were classified as non-responders to clopidogrel treatment and the remaining 243 (70%) patients were classified as responders to clopidogrel treatment. When PRU and inhibition (%) values of these two groups were compared, a high statistical difference was found for both values (respectively, $p < 0.001$, $p < 0.001$). Comparisons of the demographic and clinical characteristics of the patients are also shown in table 1. According to the statistical analysis results, there was a significant difference between the two groups only in terms of age ($p = 0.001$). The average age of the patients not responding to clopidogrel treatment was higher than those responding to the treatment. When the two groups were compared in terms of the use of beta-blocker, statin, proton pump inhibitor, calcium channel blocker and ACE inhibitor, there was no statistical difference (table 1).

Relationship between clopidogrel responsiveness and genetic variants. Twelve genetic variants which are thought to affect the pharmacokinetics and pharmacodynamics of clopidogrel were genotyped in all the patients. None of the variant alleles of *CYP2C19*4 (rs23899504)*, *CYP2C19*7* (rs72558186) and *CYP2C19*8* (rs41291556) were detected in our study group. Genotypes of other genetic variants were statistically compared according to the state of response to clopidogrel treatment (table 2). Only for the *CYP2C19*2* (G681A, rs4244285) single nucleotide polymorphism, a highly statistically significant difference was detected ($p < 0.001$). In subjects not responding to clopidogrel treatment, GA and AA genotype frequencies were high. Only three patients having the *CYP2C19*3* (G636A, rs4986893) variant were detected among the patients. One of these subjects with heterozygous genotype (GA) was in the patient group responding to clopidogrel treatment, while the other two were in the patient group not responding to the treatment. CT genotype frequency of the *CYP2C19*17* (C806T, rs12248560) single nucleotide polymorphism was found to be higher in the patients responding

Table 1.

Demographic and clinical characteristics of the study patients.

Demographic and clinical characteristics	All (n = 347)	Responder (n = 243)	Non-responder (n = 104)	*p* Value
Gender, n (%)				
Male	274 (79)	195 (80.2)	79 (76)	0.37[1]
Female	73 (21)	48 (19.8)	25 (24)	
	Median (25th–75th)	Median (25th–75th)	Median (25th–75th)	
Age, years	62 (54–70)	60 (53–69)	64.5 (58–72)	**0.001**[2]
Body mass index (kg/m^2)	27 (25–30)	27 (25–30)	27 (25–31)	0.907[2]
Serum creatinine (mg/dL)	0.97 (0.83–1.13)	0.96 (0.83–1.10)	0.97 (0.86–1.2)	0.393[2]
LDL cholesterol (mg/dL)	110 (85–137)	111 (87–139)	108.5 (83–133.7)	0.448[2]
HDL cholesterol (mg/dL)	41 (34–48)	41 (35–47)	41 (33–50.75)	0.683[2]
Platelet count, $\times 10^3/\mu L$	237 (197–277)	236 (195–279)	238 (204.5–274.2)	0.760[2]
Platelet aggregation status				
$P2Y_{12}$ reaction units (PRUs)	167.5 (120–168)	143 (101–174)	241.5 (220.2–273.5)	**<0.001**[2]
Platelet inhibition (%)	35 (24–56)	41 (29–60)	13 (5–20)	**<0.001**[2]
Risk Factors				
Hypertension, n (%)	185 (53.3)	122 (50.2)	63 (60.6)	0.076[1]
Hypercholesterolemia, n (%)	145 (41.8)	100 (41.2)	45 (43.3)	0.714[1]
Diabetes mellitus, n (%)	140 (40.3)	91 (37.4)	49 (47.1)	0.093[1]
Family history of CAD, n (%)	90 (25.9)	64 (26.3)	26 (25)	0.795[1]
Previous and current smoker, n (%)	87 (25.1)	64 (26.3)	23 (22.1)	0.406[1]
Previous PCI or CABG, n (%)	166 (47.8)	120 (49.4)	46 (44.2)	0.379[1]
Medications				
Beta-blocker, n (%)	329 (95.1)	233 (96.1)	96 (92.8)	0.322[1]
ACE inhibitor, n (%)	233 (67.3)	155 (63.6)	78 (75.4)	0.116[1]
Statin, n (%)	317 (91.5)	220 (90.9)	97 (92.8)	0.844[1]
Calcium channel antagonist, n (%)	28 (8.1)	19 (7.8)	9 (8.7)	0.819[1]
Proton pump inhibitor, n (%)	300 (86.5)	210 (86.4)	90 (87)	0.905[1]

n, number of patients; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CAD, coronary artery disease; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; ACE, angiotensin-converting enzyme.

Non-normally distributed data are presented as median (25th percentile–75th percentile). The body mass index is the weight in kilograms divided by the square of the height in metres. *p* Values indicate significance effects of demographic and clinical characteristics on the results of comparisons between the responder and non-responders group. $p < 0.05$ was considered a statistically significant difference and shown in bold.

[1]Pearson chi-square test.

[2]Mann–Whitney *U*-test.

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to clopidogrel. However, this high rate was not statistically significant ($p = 0.375$). When the genotype frequencies of *ABCB1* C3435T (rs1045642), *CYP3A4*1G* (rs2242480), *CYP2B6*9* (rs3745274), *ITGB3* L33P (rs5918), *PON1* Q192R (rs662) and *PON1* L55M (rs854560) polymorphisms were compared in the patient groups responding and not responding to clopidogrel treatment, however, there was no statistical difference (table 2). We also performed multiple logistic regression analysis including all variants in table 2 and found that the only significant variant was CYP2C19*2 (rs4244285), consistent with what appears from table 2. Because it does not have a different significant, we did not specify it as a table.

When we created a stepwise logistic regression model for the *CYP2C19*2* (G681A, rs4244285) polymorphism, when compared to ancestral GG genotype, odds ratio of GA genotype was 3.37 (95% CI 1.93–5.53), while odds ratio of AA genotype was 4.81 (95% CI: 1.47–5.53). When compared to ancestral G allele, odds ratio of A allele, on the other hand, was 2.92 (95% CI: 1.91–4.46). The other details with respect to stepwise regression model are shown in table 3.

Correlating genetic variants and pharmacodynamic response. We compared PRU and inhibition (%) values of the patients by grouping them according to their variant genotypes. There was a high difference between the PRU and inhibition (%) values of genotypes of the *CYP2C19*2* (G681A, rs4244285) polymorphism (respectively, $p < 0.001$, $p < 0.001$). The paired statistical comparisons of genotypes of the *CYP2C19*2* polymorphism in terms of PRU values are also shown in fig. 1. As we detected only six patients with TT genotype of the

Table 2.
Genotypic frequencies of the variants among the study patients according to the state of response to clopidogrel treatment.

Genetic variant and genotypes	All (n = 347)	Responder (n = 243)	Non-responder (n = 104)	*p* Value
CYP2C19*2 (G681A) (rs4244285)				
GG — n (%)	253 (72.9)	196 (80.7)	57 (54.8)	**<0.001**[1]
GA — n (%)	82 (23.6)	42 (17.3)	40 (38.5)	
AA — n (%)	12 (3.5)	5 (2.1)	7 (6.7)	
CYP2C19*3 (G636A) (rs4986893)				
GG — n (%)	344 (99.1)	242 (99.6)	102 (98.1)	0.215[2]
GA — n (%)	3 (0.9)	1 (0.4)	2 (1.9)	
AA — n (%)	0	0	0	
CYP2C19*17 (C806T) (rs12248560)				
CC — n (%)	243 (70)	165 (67.9)	78 (75)	0.388[3]
CT — n (%)	98 (28.2)	74 (30.5)	24 (23.1)	
TT — n (%)	6 (1.7)	4 (1.6)	2 (1.9)	
ABCB1 (C3435T) (rs1045642)				
CC — n (%)	88 (25.4)	60 (24.7)	28 (26.9)	0.581[1]
CT — n (%)	178 (51.3)	129 (53.1)	49 (47.1)	
TT — n (%)	81 (23.3)	54 (22.2)	27 (26)	
CYP3A4*1G (IVS10 + G12A) (rs2242480)				
GG — n (%)	252 (72.6)	181 (74.5)	71 (68.3)	0.324[3]
GA — n (%)	89 (25.6)	57 (23.5)	32 (30.8)	
AA — n (%)	6 (1.7)	5 (2.1)	1 (1)	
CYP2B6*9 (G15631T) (rs3745274)				
GG — n (%)	0	0	0	0.640[1]
GT — n (%)	243 (70)	172 (70.8)	71 (68.3)	
TT — n (%)	104 (30)	71 (29.2)	33 (31.7)	
ITGB3 (T176C) (rs5918)				
TT — n (%)	276 (79.5)	190 (78.2)	86 (82.7)	0.518[3]
TC — n (%)	64 (18.4)	47 (19.3)	17 (16.3)	
CC — n (%)	7 (2)	6 (2.5)	1 (1)	
PON1 (A575G) (rs662)				
AA — n (%)	159 (45.8)	117 (48.1)	42 (40.4)	0.200[1]
AG — n (%)	145 (41.8)	94 (38.7)	51 (49)	
GG — n (%)	43 (12.4)	32 (13.2)	11 (10.6)	
PON1 (T163A) (rs854560)				
TT — n (%)	47 (13.5)	35 (14.4)	12 (11.5)	0.466[1]
TA — n (%)	137 (39.5)	91 (37.4)	46 (44.2)	
AA — n (%)	163 (47)	117 (48.1)	46 (44.2)	

n, number of patients; CYP, Cytochrome P450; rs, the accession number of the variant in the National Center for Biotechnology Information.
The variant alleles CYP2C19*4, CYP2C19*7 and CYP2C19*8 were not present in the study population. *p* Values indicate significance of effects of genotype groups on the results of comparisons between the responder and non-responder group. $p < 0.05$ was considered a statistically significant difference and shown in bold.
[1]Pearson chi-square test.
[2]Fisher's exact test.
[3]Exact Monte Carlo chi-square test.

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Table 3.
Comparisons of genotype and allele frequencies of CYP2C19*2 (G681A) single nucleotide polymorphism between responders and non-responders to clopidogrel treatment.

Genotypes and Alleles	Responder (n = 243)	Non-responder (n = 107)	*p* Value	Odds Ratio (95% Confidence Interval)
GG — n (%)	196 (80.7)	57 (54.8)		1 (Reference)
GA — n (%)	42 (17.3)	40 (38.5)	$p < 0.001$[1]	3.27 (1.93–5.53)
AA — n (%)	5 (2.1)	7 (6.7)	$p = 0.009$[1]	4.81 (1.47–5.53)
G allele — n (%)	434 (89.3)	154 (74)		1 (Reference)
A allele — n (%)	52 (10.7)	54 (26)	$p < 0.001$[1]	2.92 (1.91–4.46)

$p < 0.05$ was considered a statistically significant difference.
[1]Stepwise binary logistic regression; n, number of patients.

*CYP2C19*17* (C806T, rs12248560), we did not statistically analyse the values of this genotype. There was a significant difference between the PRU and inhibition (%) values of CC and CT genotypes (table 4). When compared to the CC genotype, the average PRU value of the CT genotype was lower ($p = 0.029$; fig. 1), while inhibition (%) value range was higher ($p = 0.008$). There was no statistically significant difference between the genotypes of other genetic variants in terms of PRU and inhibition (%) values (table 4).

Discussion

In this study, when we grouped the patients having PRU>208 as non-responders to clopidogrel treatment, a non-responsiveness table at a rate of 30% emerged (table 1). Stone *et al.* [18] determined that PRU value of 42.7% of the 8583 patients was more than 208 and that these patients were significantly associated with definite stent thrombosis and myocardial infarction. According to the study by Stone *et al.,* the high non-responsiveness rate of clopidogrel treatment that we obtained in a small patient group indicates the severity of the condition in the Turkish population. When we compared the PRU and inhibition (%) values of the patients responding and not responding to clopidogrel, however, the finding of a high statistical difference for the two parameters is important for the homogeneity of the groups (table 1). To put it more explicitly, it indicates the sufficiency of the two groups in determining the genetic variants associated with non-responsiveness to clopidogrel.

Table 4.
Relationship between the genotypes of CYP2C19*2 and CYP2C19*17 polymorphisms and pharmacodynamic response.

	PRU[1]	Inhibition (%)[2]
CYP2C19*17 (C806T) (rs12248560)		
CC (n = 243)	172.75 ± 73.38	30 (15–48)
CT (n = 98)	153.87 ± 69.06	36 (22.75–58)
TT (n = 6)		
p Value	0.029[3]	0.008[4]
CYP2C19*2 (G681A) (rs4244285)		
GG (n = 253)	153.71 ± 71.21	35 (20–58.5)
GA (n = 82)	204.35 ± 60.97	21 (9.5–33)
AA (n = 12)	206.41 ± 67.55	19 (7.5–22.75)
p Value	<0.001[5]	<0.001[6]

n, number of patients; rs, the accession number of the variant in the National Center for Biotechnology Information.
There was no statistically significant difference between the genotypes of other genetic variants in terms of PRU and inhibition (%) values. *p* values indicate significance of effects of PRU and the inhibition (%) on the results of comparisons between the genotypes. $p < 0.05$ was considered a statistically significant difference.
[1]The results are presented as mean ± standard deviation.
[2]The results are shown as median (25th percentile–75th percentile).
[3]Student's *t*-test.
[4]Mann–Whitney *U*-test.
[5]One-way ANOVA.
[6]Kruskal–Wallis test.

It should be noted that genetic variants play a substantial role in non-responsiveness to clopidogrel treatment, but the other factors are also effective [4]. In addition to clinical and genetic factors, one of the most important reasons is the drug interaction. The drugs inhibiting CYP2C19 and CYP3A4 of the cytochrome P450 enzymes decrease the active metabolite level of serum clopidogrel [6]. Therefore, such drugs especially as omeprazole declared by FDA were not prescribed in our patient group [11]. When we compared the drug use of the patients responding and not responding to clopidogrel treatment, there was no statistical difference, which provided equal conditions between the two groups in terms of drug interaction (table 1). When we compared other clinical and demographic characteristics of the patients, age range of the subjects not responding to clopidogrel was significantly high ($p < 0.001$). Decreased hepatic bioactivation depending on more drug use, liver dysfunction and intestinal absorption disorders which are frequently seen at later ages may be among the causes of non-responsiveness in clopidogrel treatment.

The US Food and Drug Administration (FDA) declared in a Boxed Warning dated 12 March 2012 that *CYP2C19*2* and **3* alleles were devoid of functional metabolism of clopidogrel and likewise, *CYP2C19*4*, **5*, **6*, **7* and **8* alleles may be devoid of or may have a lower metabolism than clopidogrel metabolism [11]. This declaration was a result of the pharmacogenetics studies conducted until that time in Western societies [7,15,19]. However, some studies suggested that these alleles did not affect the antiplatelet activity of clopidogrel [6]. The statistical findings that we obtained in terms of both genotype and allele strongly showed that *CYP2C19*2* allele was devoid of clopidogrel metabolism in Turkish patients (table 2). In addition, the risk of non-responsiveness of the *CYP2C19*2* allele to clopidogrel treatment was found 2.92 times higher

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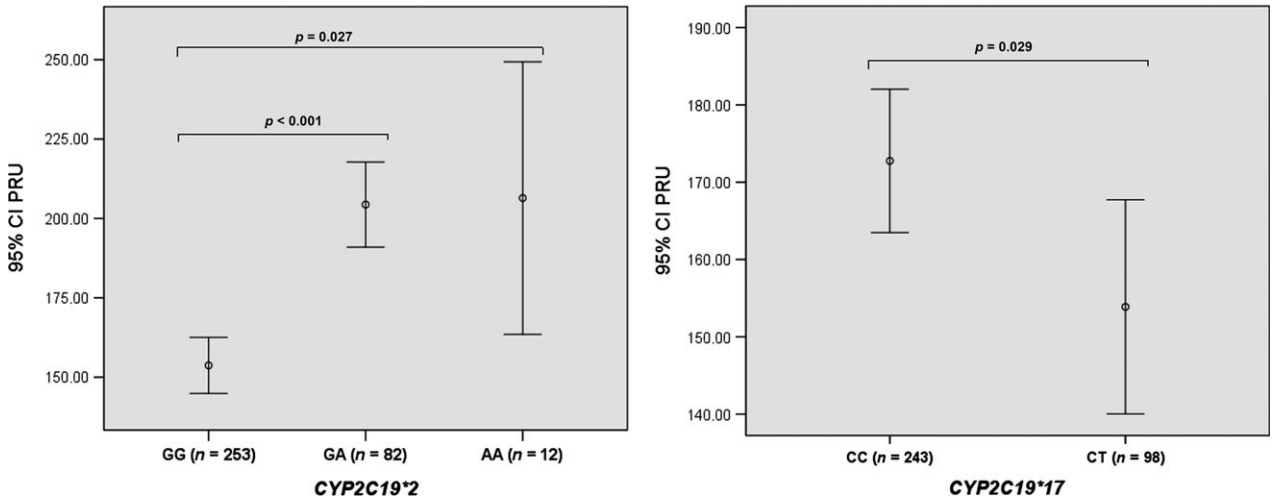


Fig. 1. VerifyNow $P2Y_{12}$ reactivity represented as error bars and grouped according to CYP2C19*2 (G681A) and CYP2C19*17 (C806T) genotypes. PRUs, platelet reactivity units.

than the ancestral type allele (table 3). FDA reported that *CYP2C19*4*, **5*, **6*, **7* and **8* alleles were in low frequency [11]. The studies conducted before and after the FDA report also reported that the frequency of each of these alleles was at a level of about 1% in the populations [15,20,21]. In our patient group, however, while *CYP2C19*4*, **7* and **8* alleles were not found at all, only three patients having the *CYP2C19*3* allele were present. When the genotype frequencies of the other genetic variants compared between the patient groups responding and not responding to clopidogrel, there was no statistical difference (table 2).

The genetic variants that we compared with respect to state of responsiveness to clopidogrel treatment based on a certain limit value may not have been correlated based on our sample size. Therefore, we analysed separately the relationship between the genetic variant genotypes and PRU and inhibition (%) values, a pharmacodynamic indicator (table 4). We confirmed again the *CYP2C19*2* (rs4244285) polymorphism that we found highly correlated with non-responsiveness to clopidogrel treatment (fig. 1). Besides, we determined that the *CYP2C19*17* (rs12248560) polymorphism significantly decreased the PRU values (fig. 1), while it increased the inhibition (%) values. Thus, it has been suggested that *CYP2C19*17* (rs12248560) polymorphism, which is not correlated according to the PRU>208 limit value, may play an important role in clopidogrel metabolism. The previous studies also showed the decreasing effect of the *CYP2C19*17* (rs12248560) polymorphism both for major cardiovascular events [22] and platelet reactivity values [23]. Despite these results, the effect of the allelic variant (rs12248560) on clopidogrel antiplatelet activity is still controversial. Yet, the latest meta-analysis studies strengthen that it is a gain-of-function mutation [24].

Clopidogrel is a substrate of the efflux P-glycoprotein, which is encoded by the *ABCB1* gene [20]. The correlation of C3435T single nucleotide polymorphism in *ABCB1* gene with clopidogrel response is still controversial. While some studies associated this polymorphism with the low response to clopidogrel treatment [13,20,21,25], others suggested that such an association was not available [12,26–28]. We did not find a correlation of both response to clopidogrel treatment and antiplatelet activity with the *ABCB1* C3435T (rs1045642) polymorphism.

CYP3A4 metabolic activity correlated with the variability in the platelet inhibition in patients after clopidogrel [29]. Angiolillo *et al.* [14] reported that among patients using clopidogrel, those carrying allele of the *CYP3A4* IVS10 + 12G>A (rs2242480) polymorphism had increased platelet inhibition. Jinnai *et al.,* however, did not find any correlation between the *CYP3A4* IVS10 + 12G>A (rs2242480) polymorphism and platelet aggregation inhibition [30]. Our results also supported the study by Jinnai *et al.*

Mega *et al.* [15] detected that among the patients treated with clopidogrel, those carrying the allele of the *CYP2B6*9* (rs3745274) polymorphism had lower active metabolite level and platelet aggregation inhibition. However, Viviani Anselmi *et al.* [31] reported that the *CYP2B6*9* (rs3745274) polymorphism was not correlated with on-treatment platelet reactivity (OTR) and predicting major adverse cardiac events (MACE). Our antiplatelet activity results was corroborating with the study by Viviani Anselmi *et al.*

Glycoprotein (GP) IIb/IIIa complex on the platelet cell membrane is bound to fibrinogen and von Willebrand factor and plays an important role in platelet reactivity. Firstly, it was reported that the L33P polymorphism (rs5918) in *ITGB3* gene encoding GPIIIa subunit of this complex increased the GPIIb/IIIa activation of and reduced the antiplatelet effect of clopidogrel [16]. Subsequent studies, however, reported that *ITGB3* L33P (rs5918) polymorphism was not correlated either with antiplatelet activity [32] or with ischaemic events [20]. We, on the other hand, did not find a significant correlation when we compared *ITGB3* L33P (rs5918) polymorphism with the response to clopidogrel treatment and antiplatelet activity values.

In the study by Bouman *et al.*, they used *in vitro* metabolomic profiling techniques providing a different point of view to the pharmacogenetics of clopidogrel. They reported that PON1

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is important for bioactivation of clopidogrel and PON1 Q192R (rs662) polymorphism determines the active metabolite formation rate of clopidogrel [12]. Afterwards, while Li *et al.* supported this study [33], others rejected the presence of such an association [34–36]. Due to the findings in conflict with each other, the doubt on PON1 has not been removed yet. Therefore, in our study, we analysed PON1 Q192R (rs662) polymorphism by also including L55M (rs854560) polymorphism, which is common and functional in the populations therein. There was no significant correlation between antiplatelet activity values and response to clopidogrel treatment with respect to the genotypes of the two polymorphisms.

The reason for not finding a correlation between the variants other than the *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) polymorphisms and the response to clopidogrel treatment may have resulted from the multiple proteins having a role in antiplatelet activity formation. Moreover, the presence of unpredictable clinical factors leading to the development of non-responsiveness may also be among the causes.

Although new antithrombotic agents (prasugrel, ticagrelor) have gradually come to the forefront in order to form an effective antiplatelet activity, clopidogrel is still commonly used around the world. Increased risk of bleeding due to new generation agents and high selling prices show that clopidogrel will maintain its importance in antiplatelet treatment.

We confirm a statistically significant effect of *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) variant genotype on clopidogrel pharmacodynamics as measured by platelet aggregation. Our findings support a likely clinically relevant effect of *CYP2C19*2*.

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Conflict of interest

The authors report no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Primers for the amplification and extension PCR reactions designed in the Sequenom Assay Designer 3.1 (Sequenom, San Diego, CA, USA) software.

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