

INFLUENCE OF CYP2C19*2 GENE VARIANT ON THERAPEUTIC RESPONSE DURING CLOPIDOGREL TREATMENT IN PATIENTS WITH CAROTID ARTERY STENOSIS

UTICAJ CYP2C19*2 VARIJANTE GENA NA TERAPIJSKI ODGOVOR U TOKU PRIMENE KLOPIDOGRELA KOD BOLESNIKA SA STENOZOM KAROTIDNE ARTERIJE

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Summary

Background: Despite the proven clinical effect of oral antiplatelet drugs, a considerable number of patients do not have an adequate response to clopidogrel. The aim of our study was to determine the influence of CYP2C19*2 loss-of-function variant allele on clopidogrel responsiveness in patients with carotid artery stenosis.

Methods: One hundred and twelve patients with carotid artery stenosis undergoing endarterectomy were included in this one-year prospective study. All of them received clopidogrel (75 mg daily) for at least 30 days after the intervention. They were followed from the moment of hospital admission. CYP2C19*2 genotyping was performed by TaqMan Assay. The influence of CYP2C19*2 variant allele on clopidogrel platelet reactivity was determined using multiple-electrode aggregometry (MEA).

Results: Genotyping results showed that 82 (73.2%) patients were homozygous for wild type, 29 (25.9%) were heterozygous for the CYP2C19*2 allele and 1 (0.9%) was CYP2C19*2 homozygous. After 24 hours, among those with the wild type 29.3% were clopidogrel responders, and in those with the CYP2C19*2 alleles 10%. In the wild type group, 74.4% were clopidogrel responders after 7 days of

Kratik sadržaj

Uvod: I pored dokazanog kliničkog efekta oralne antiagregacijske terapije, značajan broj pacijenata nema adekvatan odgovor na primenjeni klopidogrel. Cilj naše studije je bio da se utvrdi uticaj prisutne CYP2C19*2 varijante gena na terapijski odgovor u toku primene klopidogrela kod pacijenata sa stenozom karotidne arterije.

Metode: U jednogodišnju prospektivnu studiju uključeno je 112 pacijenata sa stenozom karotidne arterije kod kojih je izvršena endarterektomija. Posle operativnog zahvata, pacijenti su primali 75 mg dnevno klopidogrela u trajanju od najmanje mesec dana. Svi ispitanici su praćeni od momenta prijema. Za CYP2C19 genotipizaciju korišćen je TaqMan test. Uticaj CYP2C19*2 alela na trombocitnu reaktivnost ispitivan je primenom *multiple-electrode aggregometry* (MEA).

Rezultati: Rezultati genotipizacije su pokazali da su 82 (73,2%) ispitanika homozigoti za *wild-type*, 29 (25,9%) heterozigoti za CYP2C19*2 alel, dok je 1 (0,9%) bio homozigot za CYP2C19*2. Nakon 24 sata, u grupi sa *wild-type* genotipom 29,3% ispitanika dali su odgovor na klopidogrel, a u grupi sa CYP2C19*2 varijantom gena 10% ispitanika. U grupi sa *wild-type* genotipom, 74,4% ispitanika su imali terapijski odgovor nakon 7 dana, odnosno 82,9%

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Abbreviations: CYP (the hepatic cytochrome P450), PCI (percutaneous coronary intervention), CYP2C19*2; CYP2C19*3 (genetic polymorphism), MEA (multiple electrode aggregometry), AUC (area under curve), PCR (polymerase chain reaction), BMI (body mass index), HDL (high density lipoprotein), CEA (carotid endarterectomy), CCBs (calcium channel blockers).

taking the drug; 82.9% after 30 days of clopidogrel introduction, respectively. In patients with the *CYP2C19*2* alleles the number of responders increased up to 46.7% after 7 days; 53.3% after 30 days of taking the drug, respectively. The risk for being a low-responder is higher for the patients heterozygous for the *CYP2C19*2* allele vs. wild-type (OR 4.250, 95% CI 1.695–10.658, $P < 0.01$).

Conclusions: The *CYP2C19*2* loss-of-function variant allele has significant influence on clopidogrel response in patients with carotid artery stenosis undergoing endarterectomy.

Keywords: clopidogrel, *CYP2C19*2*, platelet reactivity

Introduction

Platelets play a key role in the development of thrombotic complications in patients with atherosclerotic vascular disease (1). Oral antiplatelet drugs, aspirin and clopidogrel are the cornerstone therapy in conditions characterized by risk for arterial thrombosis (1). They are widely used as treatment for patients with acute coronary syndrome and for stent thrombosis prevention in patients undergoing percutaneous coronary intervention (2). Regarding patients undergoing carotid endarterectomy, perioperative anti-thrombotic therapy should consist of aspirin alone, while the addition of clopidogrel should be considered for high risk patients (3).

Despite their proven clinical effect, a considerable number of patients do not have an adequate response to aspirin, clopidogrel or both (4). Ten to fifteen percent of patients receiving these medications have recurrent atherothrombotic events while receiving treatment (2, 5).

Clopidogrel is a second-generation oral thienopyridine. It is a prodrug which is converted into its active metabolite by the isoforms of hepatic cytochrome P450 (CYP) in a two-step process. The active metabolite irreversibly inhibits ADP-stimulated platelet aggregation covalently binding to the purinergic P2Y₁₂ receptor on the platelet surface (2). Approximately 85% is hydrolyzed into an inactive carboxylic acid derivative compound by esterase and 15% of the administered clopidogrel form the active clopidogrel thiol metabolite. The *CYP2C19* enzyme is involved in both oxidative steps of clopidogrel metabolism (2, 4). Several pharmacogenomic studies demonstrated that *CYP2C19* variant alleles affect the pharmacokinetics and pharmacodynamics of clopidogrel and influence the interindividual heterogeneity of the clopidogrel response (2). Variant alleles for which reduced or loss of function have been shown comprise *CYP2C19*2* to *CYP2C19*8*. The *CYP2C19*2* gene variant is a G681A mutation in exon 5 which encodes for a cryptic splice variant, while a G636A mutation in exon 4 results in a premature stop codon in *CYP2C19*3* (6). The *CYP2C19*2* is the most

after 30 days of primene klopidoğrela. U grupi sa *CYP2C19*2* alelom broj ispitanika sa terapijskim odgovorom raste do 46,7% nakon 7 dana, odnosno do 53,3% nakon 30 dana od primene klopidoğrela. Rizik za slab odgovor je veći kod nosilaca *CYP2C19*2* alela u odnosu na nenosiocce (*wild-type*) (OR 4,250, 95% CI 1.695–10.658, $P < 0,01$).

Zaključak: *CYP2C19*2* varijanta gena značajno utiče na terapijski odgovor u toku primene klopidoğrela kod bolesnika sa stenozom karotidne arterije kod kojih je izvršena endarterektomija.

Ključne reči: klopidoğrel, *CYP2C19*2* varijanta gena, reaktivnost trombocita

frequent variant allele in the Caucasian and the African population while *CYP2C19*3* to *CYP2C19*8* alleles have minor clinical impact due to their low frequency (<1%) (6). Individuals who are carriers of *CYP2C19*2* and *CYP2C19*3* alleles have a reduced metabolism of clopidogrel into its active metabolite and reduced ex vivo platelet reactivity, which leads to an increased risk of adverse cardiovascular events (5). The *CYP2C19*17* allelic variant, a C806T mutation in exon 5, is responsible for an increased catalytic activity. Carriage of *CYP2C19*17* allele(s) results in an increased platelet inhibition by clopidogrel due to an enhanced formation of the active metabolite which could lead to an increased risk of bleeding (6). So far, studies have examined whether clopidogrel treatment is affected by *CYP2C19* variant alleles in patients with acute coronary syndrome (7, 8), myocardial infarction (9), coronary artery disease (10, 11) and in patients undergoing percutaneous coronary intervention (PCI) (12–16).

According to the published data, the presence of *CYP2C19*2* variant allele(s) leads to a reduction in clopidogrel-induced platelet inhibition. The aim of our study was to determine the influence of *CYP2C19*2* loss-of-function variant allele on clopidogrel responsiveness in patients with carotid artery stenosis undergoing endarterectomy.

Materials and Methods

Patients

From June 2012 to October 2013, 112 consecutive patients with carotid artery stenosis undergoing elective endarterectomy (CEA) at Institute for Cardiovascular Diseases »Dedinje«, Belgrade, were enrolled in the prospective study. The patients' characteristics are show in *Table I*.

Related to the study criteria, all participants who received clopidogrel (75 mg daily) for at least 30 days from intervention were eligible for the study. Exclusion criteria were renal and/or liver dysfunction, diabetes mellitus, preoperative platelet count below $150 \times 10^9/L$, intake of antiplatelet drugs other than

Table I Baseline characteristics of patients.

Age, years (mean \pm SD)	66.2 \pm 8.13
Gender, n (%)	
Male	66 (58.9)
Female	46 (41.1)
BMI, n (%)	
< 30	91 (81.3)
> 30	21 (18.8)
Risk factors, n (%)	
Smoking status/yes	84 (75.0)
Hypercholesterolemia/yes	40 (35.7)
Hypertriglyceridemia/yes	26 (23.2)
Positive family history of cardiovascular disease/yes, n (%)	41 (36.6)
Presence of stenosis of both ACI, n (%)	71 (63.4)
Previous stroke or TIA, n (%)	40 (35.7)
Stroke/yes	33 (82.5)
TIA/yes	7 (17.5)
Laboratory data (mean \pm SD)	
Fibrinogen, g/L	4.6 \pm 1.1
Leukocytes, 10 ⁹ /L	7.0 \pm 1.9
Hemoglobin, g/L	136.5 \pm 12.3
Platelets, 10 ⁹ /L	219 \pm 57
Co-medication, n (%)	
Aspirin	112 (100)
Beta blocker	41 (36.6)
ACE inhibitor	64 (57.1)
Calcium channel blocker	35 (31.3)
Angiotensin II receptor antagonist	4 (3.6)
Statins	53 (47.3)

SD (standard deviation), BMI (body mass index, kg/m²), CVD (cardiovascular diseases), ACI (internal carotid artery), TIA (transitory ischemic attack), ASA (acetylic salicylic acid), ACE (angiotensin converting enzyme).

aspirin and clopidogrel. On admission patients were initially treated with aspirin. During their hospital stay after elective CEA, in all of them a dual combination of aspirin and clopidogrel of 75 mg was continued. The patients were followed up for 12 months from the moment of hospital admission. Patient compliance was assessed by telephone interviews. The outcomes that were evaluated during follow up were TIA, stroke and death.

The research protocol was approved by the Ethics Committee of the Institute for Cardiovascular Diseases »Dedinje«. All patients gave written informed consent for genotype determination and platelet function testing prior to study inclusion.

Blood sampling

For whole blood impedance aggregometry, blood samples were collected in tubes containing heparin as anticoagulant (BD, Swingdon, UK). Blood samples were kept at room temperature for approximately 30 min before platelet function testing. For genetic testing, blood samples were collected in tubes containing sodium citrate as anticoagulant (BD, Swingdon, UK).

Platelet function testing

For platelet function testing multiple electrode aggregometry (MEA) is used, which has been performed using the impedance aggregometer (Multiplate analyzer, Roche Diagnostics, Mannheim, Germany). ADP-induced platelet aggregation was used to analyze the effect of clopidogrel. The instrument continuously measures the impedance change, which is proportional to the amount of platelet aggregates on the electrode wires. The area under the aggregation curve (AUC) was used to express the aggregation response over the measured time (AU*min).

Platelet aggregation was measured three times after intervention: 24 h after the first dose (75 mg) of clopidogrel, after 7 days and 30 days of taking the drug. In relation to the published results (17, 18), we performed the first measurement after 24 hours of clopidogrel in order to determine whether a lower clopidogrel dose (75 mg) affected platelet aggregation enough to point the difference in respect to the CYP2C19*2 carriers status.

We prospectively defined low response to clopidogrel by setting a cutoff point at the upper quartile (25%) of measurements by MEA 30 days after clopidogrel introduction. The pointed cutoff value of 575 AU*min was used in the assessment of therapeutic response at all three time points of measurements.

Genotyping

Genomic DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the supplier's instruction. Genotyping for CYP2C19*2 variant allele was performed by TaqMan Drug Metabolism Genotyping Assay for SNP rs4244285 (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA, US), on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The results were analyzed by Applied Biosystems 7500 Real-Time PCR System Sequence Detection Software v1.4.1.

Statistical methodology

The normality of continuous variables was evaluated using the Kolmogorov-Smirnoff test. According to the Kolmogorov-Smirnoff test, all data showed normal distribution and continuous variables are presented as mean and standard deviation (SD). Categorical variables are presented as numbers or percentages and they were compared using Pearson chi-square test.

Testing of the difference in ADP values at three different moments and in two groups was performed using two way parametric ANOVA with the Bonferroni test for *post hoc* comparison. Finally, for estimating predictors of low responsiveness, the binary logistic regression model was used.

A P value < 0.05 was considered to indicate a significant difference. Statistical analyses in all applied methods were performed using SPSS version 20.0 (SPSS Inc., Chicago, Illinois).

Results

Genotyping results showed that 82 (73.2%) patients were homozygous for wild type, 29 (25.9%)

were heterozygous for the *CYP2C19*2* variant allele and 1 (0.9%) was homozygous for *CYP2C19*2*. Thus, the wild type genotype frequency was 73.2% vs. 26.8% for *CYP2C19*2* carriers (Table II).

Mean and SD values of ADP-induced platelet aggregation across *CYP2C19* genotypes measured 24 h after the first dose of clopidogrel, after 7 and 30 days of taking the drug are presented in Figure 1. The values of platelet aggregation were statistically significantly different between the two genotype groups, $P < 0.01$. As showed in the Figure 1, platelet aggrega-

Table II Prevalence of *CYP2C19* genotypes in study population.

Genotype	n (%)
Wild type (<i>CYP2C19*1/*1</i>)	82 (73.2)
Heterozygous for <i>CYP2C19*2</i>	29 (25.9)
Homozygous for <i>CYP2C19*2</i>	1 (0.9)

CYP (the hepatic cytochrome P 450)

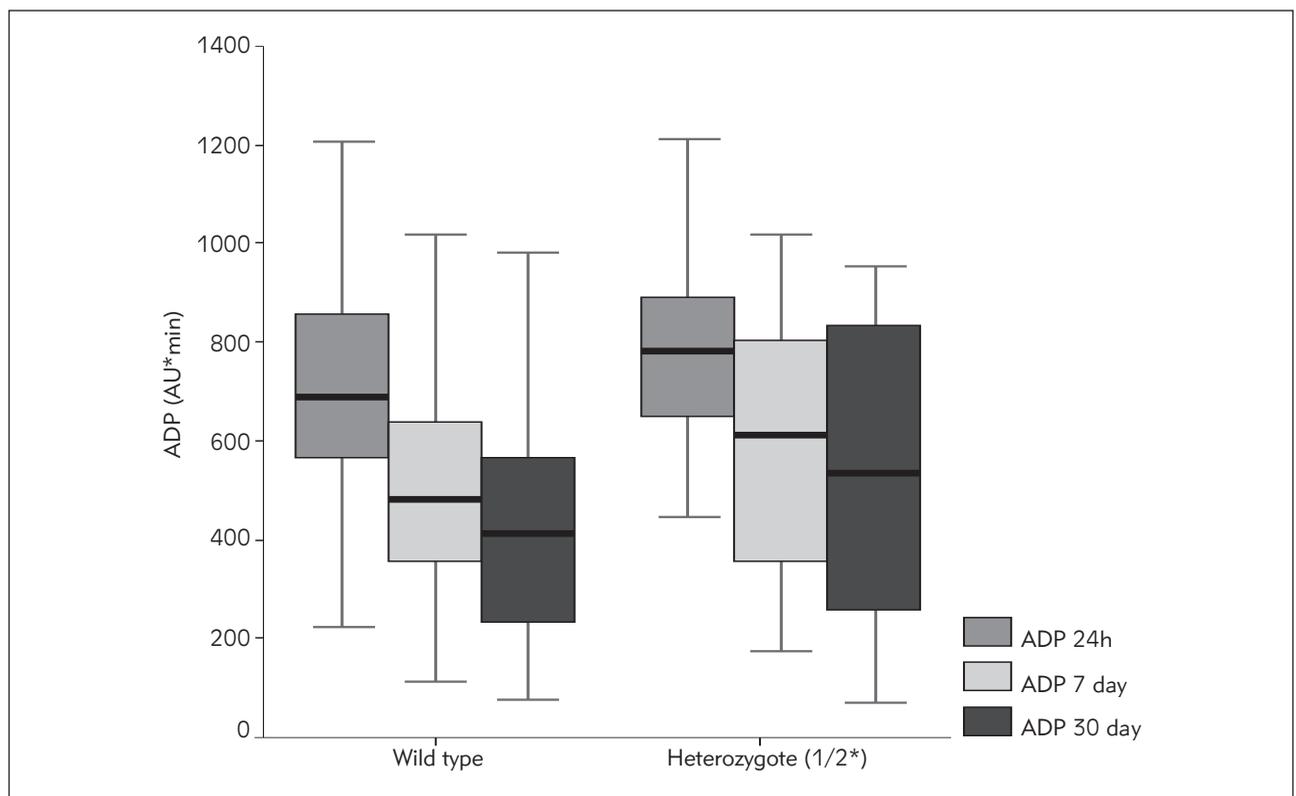


Figure 1 ADP-induced platelet aggregation across *CYP2C19* genotypes measured 24 h after the first clopidogrel dose, after 7 days and after 30 days of clopidogrel treatment.

The bottom of the box plots for ADP-induced platelet inhibition mark the 25th percentile, the median lines mark the 50th percentile, the top of the boxes mark the 75th percentile, the bottom and top of the vertical lines mark the 5th and 95th percentile.

Table III ADP-induced platelet aggregation values over time across CYP2C19 genotypes.

	ADP 24h, mean (SD)#	ADP 7day, mean (SD)#	P value
Wild type	672.28 (189.71)	474.63 (191.30)	<0.001
Heterozygous for CYP2C19*2	763.00 (165.08)	598.80 (232.18)	0.001
	ADP 7day, mean (SD)#	ADP 30 day, mean (SD)#	P value
Wild type	474.63 (191.30)	389.10 (183.47)	<0.001
Heterozygous for CYP2C19*2	598.80 (232.18)	560.50 (257.06)	0.241
	ADP 24h, mean (SD)#	ADP 30 day, mean (SD)#	P value
Wild type	672.28 (189.71)	389.10 (183.47)	<0.001
Heterozygous for CYP2C19*2	763.00 (165.08)	560.50 (257.06)	<0.001

#Platelet aggregation was expressed as AU*min

Table IV Correlation between genotype and phenotype.

After 24 hours of taking the drug			P value
	Wild type	Heterozygous for CYP2C19*2	0.036
clopidogrel responder, n (%)	24 (29.3)	3 (10.0)	
clopidogrel low-responder, n (%)	58 (70.7)	27 (90.0)	
After 7 days of taking the drug			
	Wild type	Heterozygous for CYP2C19*2	0.006
clopidogrel responder, n (%)	61 (74.4)	14 (46.7)	
clopidogrel low-responder, n (%)	21 (25.6)	16 (53.3)	
After 30 days of taking the drug			
	Wild type	Heterozygous for CYP2C19*2	0.001
clopidogrel responder, n (%)	68 (82.9)	16 (53.3)	
clopidogrel low-responder, n (%)	14 (17.1)	14 (46.7)	

tion was significantly higher in patients carrying the CYP2C19*2 allele than in the group of non-carriers in all three measurements; $P < 0.01$.

Analysis of the numerical values of platelet aggregation also showed significant difference in relation to genotype. In the group with wild type, ADP-induced platelet aggregation showed a tendency of decline over time. In patients with the CYP2C19*2 allele, the decline of platelet aggregation was observed after 7 days of clopidogrel and remained largely unchanged at the last measurement performed after 30 days of clopidogrel intake (Table III).

The correlation between CYP2C19 genotype and therapeutic response to clopidogrel was showed in Table IV. The cutoff value for post-treatment measurements defining the upper quartile (25%) of patients was 575 AU*min. According to this cutoff value, after 24 hours, in the group with the wild type 29.3% of patients were clopidogrel responders, compared to only 10% from the group with the CYP2C19*2 variant, $P = 0.036$. In the wild type group, 74.4% were clopidogrel responders after 7 days of taking the drug; 82.9% after 30 days of clopidogrel introduction, respectively. In patients with the

*CYP2C19*2* alleles the number of responders increased up to 46.7% after 7 days; 53.3% after 30 days of taking the drug, respectively. So, we notice a significant difference between the two genotype groups at the last two measurements of platelet reactivity, $P=0.006$, $P=0.001$ respectively.

The patient who was homozygous for *CYP2C19*2* had no therapeutic response from the beginning of the treatment. The risk for being a low-responder is higher for subjects heterozygous for the *CYP2C19*2* allele vs. wild type (OR 4.250, 95% CI 1.695–10.658). In the logistic regression model, the *CYP2C19*2* variant allele was found to be an independent predictor of low responsiveness to clopidogrel, $P<0.01$.

In the follow up period, stroke occurred in 2 patients (1.8%). Both of them are carriers of the *CYP2C19*2* allele. Due to the size of the group who developed stroke, we could not use any appropriate statistical method in order to test whether there is an influence of the *CYP2C19*2* variant allele on the development of stroke.

Discussion

To the best of our knowledge, this is the first study in which the laboratory response to clopidogrel was assessed to evaluate the relationship between the *CYP2C19*2* loss-of-function allele and platelet aggregation in patients with carotid artery stenosis undergoing CEA, who received antiplatelet treatment with clopidogrel.

In our study group, 26.8% of patients are *CYP2C19*2* carriers, which is in line with the previously published data where 27.1% of patients were carriers of at least one variant allele (6). We have to note that our data are the first results relating to the *CYP2C19*2* variant allele frequency in a Serbian population. The results from our study demonstrated that patients carrying the *CYP2C19*2* allele had significantly higher ADP-induced platelet aggregation compared to the non-carriers. This is in accordance with the results from previously published studies, which gave evidence of a relationship between *CYP2C19* variant alleles and reduced levels of the active clopidogrel metabolite in the plasma (7, 10) and reduced platelet inhibition (7, 9, 10, 12–14, 19–22) which cause poor or absence of responsiveness to clopidogrel and inadequate protection from ischemic events (7, 9, 10, 13, 14).

Analysis of the numerical values of platelet aggregation also showed significant difference in relation to genotype. Specifically, in the group with wild type ADP-induced platelet aggregation showed a tendency of decline over time, while in the group with the *CYP2C19*2* variant allele the decline in value of platelet aggregation was observed 7 days after clopidogrel treatment and remained largely unchanged.

In our study population, one patient was homozygous for *CYP2C19*2* and he had no therapeutic response from the beginning of clopidogrel treatment. Our results agree with data reported by others, where platelet aggregation values in *CYP2C19*2/*2* patients are significantly higher than in *CYP2C19*1/*2* patients and the wild type genotype (13, 19).

In our study among patients carrying the *CYP2C19*2* allele, 46.7% were clopidogrel low-responders after 30 days of taking the drug. In contrast, only 27% of patients with the loss-of-function polymorphism were classified as clopidogrel low-responders in the Pegasus-PCI study (16). Unlike the Pegasus-PCI study, our study included a smaller number of patients with different clinical presentation who were administered a lower dose of clopidogrel (75 mg). Also, a possible reason for the difference between Pegasus-PCI and our study arises from the fact that the interval between clopidogrel intake and blood sampling was different. Additionally, PCI leads to the release of multiple coagulation mediators, which additionally activate platelets (16).

Statistical analysis of our results showed that the risk for being a clopidogrel low-responder is 4.25-fold higher for patients heterozygous for the *CYP2C19*2* allele compared to those homozygous for the wild type. Similar results were presented in previous published studies, too (19, 21, 23).

We have to note that there are contrasting conclusions in the literature about the predictive value of *CYP2C19*2* variant allele as well as linking pharmacodynamic response to the drug and clinical outcomes. These uncertainties are likely due to the inaccuracies and lack of standardization of platelet functional tests that have been used to detect low-responders to clopidogrel, as well as the absence of a universally accepted and validated cutoff value for platelet function (24). Also, it has been well documented that platelet reactivity is influenced by clinical presentation and that choice of the best cutoff value is disease specific. This approach would have allowed a more accurate identification of low-responders to clopidogrel and prediction of cardiovascular outcomes during treatment (15, 24).

Further, we should stress the possible effect of co-medications on clopidogrel response. The data concerning statin–clopidogrel interactions are inconsistent. *In vitro* studies have showed that the conversion of clopidogrel into the active metabolite is inhibited by atorvastatin, but *in vivo* studies in healthy volunteers could not confirm an interaction between lipophilic statins and clopidogrel, especially when evaluations were performed with patients receiving maintenance therapy or after they had been given a 600 mg loading dose of clopidogrel (26–28). Moreover, a recent study showed that the addition of high-dose atorvastatin (80 mg) for 30 days significantly improved the pharmacodynamic effects of double-

dose clopidogrel, reducing platelet reactivity and improving optimal clopidogrel response in statin naïve patients with high-on treatment platelet reactivity on standard-dose clopidogrel (28). Some calcium channel blockers (CCBs) have strong inhibitory effects on the drug transporter p-glycoprotein (P-gp), which may cause decreased intestinal efflux of clopidogrel (29). Harmsze et al. evaluated the effect of co-administration of Pgp-inhibiting CCBs (verapamil, nifedipin, diltiazem, barnidipine) and non-Pgp-inhibiting CCBs (amlodipine) on clopidogrel platelet reactivity in patients on dual antiplatelet therapy undergoing PCI. According to the results, only amlodipine was associated with a high risk of clopidogrel poor response (30).

Among the limitations of our study that should be discussed is the fact that this is an observational single-center study that involved a relatively small number of patients and as such it should be considered as a pilot study. Also, in order to obtain clear results about the influence of CYP2C19*2 loss-of-function genotype on clopidogrel responsiveness, we

did not include the patients with diabetes mellitus. In addition, we assessed only the impact of one allelic variant of the CYP2C19 isoenzyme on clopidogrel responsiveness.

Conclusion

The results from our study showed that the CYP2C19*2 loss-of-function variant allele has significant influence on clopidogrel response in patients with carotid artery stenosis undergoing endarterectomy.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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