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The influence of genetic polymorphisms of CYP2C19 and ABCB1 on ADP-induced platelet aggregation in clopidogrel-treated patients: A comparison between the index hospitalization for myocardial infarction and the 3-month follow-up visit

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ABSTRACT

Background. Recent studies suggest that polymorphisms of genes involved in the clopidogrel metabolism may be associated with an impaired drug bioactivation and possibly with unfavourable clinical outcomes. The aim of this study was to assess the effect of selected genetic polymorphisms on adenosine diphosphate-induced platelet aggregation (ADP-PA) during the index hospitalization and after 3 months of clopidogrel therapy in patients presenting with myocardial infarction (MI).

Materials and methods. The study was designed as a single-center cohort trial with the 3-month follow-up. Genotyping for CYP2C19*2, CYP2C19*17, ABCB1 alleles and platelet reactivity assessment using the Multiplate Analyzer were performed in 157 patients.

Results. ADP-PA during the index hospitalization was significantly higher than at the 3-month follow-up visit regardless of the genotyp e [CYP2C19*1/*1 alleles (24.0 v. 15.5 U; $p < 0.00001$), CYP2C19*17 allele (CT: 25.0 v. 15.0 U; $p = 0.000\,2$; TT: 35.0 v. 22.0 U; $p = 0.02$) and ABCB1 allele (CC: 27.0 v. 15.0 U; $p < 0.0002$; CT 24.0 v. 17.0 U; $p < 0.0005$)]. In univariate analysis we failed to demonstrate any impact of the analyzed genetic variants on both in-hospital and 3-month ADP-PA, except for CYP2C19*17/*17 homozygotes. Significantly higher values of ADP-PA were found in CYP2C19*17/*17 (TT homozygotes) allele carriers when compared with carriers of two wild alleles during the index hospitalization (CC: 20.0 U v. TT: 35.0 U; $p = 0.02$), but not at the 3-month follow-up visit. Multivariate regression analysis revealed increased mean platelet volume ($\beta = 7.2$), elevated platelet count ($\beta = 0.2$) and the presence of heart failure at discharge ($\beta = 6.9$), but not genetic polymorphisms, to be independent determinants of high ADP-PA during the index hospitalization. Similarly, elderly age ($\beta = 3.3$), high white blood cell count ($\beta = 1.4$), elevated platelet count ($\beta = 0.4$) and increased mean platelet volume ($\beta = 0.1$), but not genetic polymorphisms, were independently associated with the higher values of ADP-PA after 3 months of clopidogrel therapy.

Conclusions. On-clopidogrel platelet reactivity significantly decreases beyond the acute phase of MI regardless of the genotype. Additionally, our study indicates that in clopidogrel-treated MI patients genetic polymorphisms are not the major determinants of the interindividual variability in platelet reactivity. However, due to a limited sample size, their minor contribution cannot be excluded.

Key words: clopidogrel, genetic polymorphism, myocardial infarction, platelet reactivity

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Introduction

Dual antiplatelet therapy composed of aspirin and a P2Y12 receptor inhibitor remains the mainstay of the treatment in patients with acute coronary syndromes (ACS) and those undergoing percutaneous coronary interventions (PCI). Clopidogrel, P2Y12 receptor inhibitor most commonly used in Poland and in most of the countries worldwide, when added to the aspirin therapy, proved to maintain stent patency after PCI with a favourable safety profile and to protect ACS patients from ischemic events [1–3]. In the setting of elective PCI, clopidogrel is considered the indisputable standard of care (class of recommendation I, level of evidence A), while in ACS patients clopidogrel is recommended when new agents, ticagrelor and prasugrel, are unavailable or contraindicated (class of recommendation I, level of evidence B) [4].

Although the introduction of clopidogrel to the cardiovascular armamentarium is regarded as a great advance, therapy with this drug is flawed by several limitations, with a large interindividual variability in response to clopidogrel being the most important [5]. The topic of high on-clopidogrel platelet reactivity has been recently widely discussed in the literature [6–9]. It is estimated that 5–10% of patients do not respond to clopidogrel therapy, and approximately another 25% respond to the drug incompletely [10]. This substantial variability in the treatment response may be associated with polymorphisms of the genes encoding the hepatic cytochrome P450, since clopidogrel is an inactive prodrug that requires two-step bioactivation [11, 12]. Additionally, recent studies not only suggest that polymorphisms of the genes involved in clopidogrel metabolism may be associated with an impaired drug bioactivation but also possibly with unfavourable clinical outcomes [13, 14]. Twenty five genetic variants encoding the CYP2C19 enzyme have been reported in the literature [15]. The normal activity of the CYP2C19 enzyme is determined by the presence of two wild alleles. Some of the allelic variants, particularly CYP2C19*2, lead to the complete loss of the CYP2C19 enzymatic activity, while others (e.g. CYP2C19*17) enhance clopidogrel bioactivation [16–20]. Another genetic cause of variable on-clopidogrel platelet reactivity may be the polymorphism of ABCB1, the gene encoding the P-glycoprotein involved in the clopidogrel absorption. The genetic variant 3435C>T is associated with an impaired function of the intestinal drug-efflux transporter and a reduced concentration of clopidogrel active metabolite [21]. The results of a genetic sub-analysis of the TRITON-TIMI 38 trial suggest that clopidogrel-treated 3435C>T homozygotes may be exposed to an increased risk of recurrent ischemic events [22]. Causes of the high on-clopidogrel platelet reactivity include not only genetic factors, but may also be a consequence of non-compliance, drug–drug interactions, increased platelets turnover and many clinical variables like diabetes, heart failure, older age, obesity, chronic kidney disease, hypercholesterolemia and inflammation [10, 23, 24].

The aim of this study was to assess the effect of genetic polymorphisms on ADP-induced platelet aggregation (ADP-PA) in patients presenting with myocardial infarction and after 3 months of clopidogrel therapy.

Materials and methods

Study population and design

The present study was designed as a cohort trial with the 3-month follow-up. Patients admitted to the Department of Cardiology and Internal Medicine, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz and treated with primary PCI for acute myocardial infarction were screened for eligibility. All patients received a 600 mg clopidogrel loading dose, followed by a 75 mg maintenance dose once daily in conjunction with aspirin (a 300 mg loading dose, followed by a 100 mg maintenance dose once daily).

Exclusion criteria were defined as follows:

— the need for prolonged use of oral anticoagulant therapy, heparin or fondaparinux;
— bleeding disorders (including thrombocytopenia $< 100 \times 10^3/\mu L$);
— anemia (hemoglobin < 10.0 g/dL);
— active inflammation;
— heart failure class III and IV according to the New York Heart Association (NYHA) classification;
— life expectancy < 1 year.

During the index hospitalization (on the 4th day after the admission at 10:00 a.m.) and at the 3-month follow-up visit, ADP-PA was assessed. Blood samples for genetic testing were collected on the 5th day. Genotyping was performed at the Institute of Molecular and Forensic Genetics, Collegium Medicum of the Nicolaus Copernicus University in Bydgoszcz, Poland. The study protocol was approved by the Ethical Committee of the Nicolaus Copernicus University.

Platelet reactivity assessment

Blood samples were collected into hirudin-containing tubes (Roche Diagnostics International Ltd, CH-6343 Rotkreuz, Switzerland). The 4th day of the index hospitalization was chosen for the blood sampling because at this time the patient with myocardial infarction is usually mobile, usually leaves the coronary care unit, and both aspirin and clopidogrel reach a steady state. ADP-PA was determined in the whole blood using the multiple electrode aggregometry (MEA)

on a new generation impedance aggregometer (Multiplate Analyzer, Roche Diagnostics International Ltd, CH-6343 Rotkreuz, Switzerland) according to the manufacturer's instructions. The principle of MEA is based on the fact that the platelets get sticky upon activation, and, therefore, have a tendency to adhere and aggregate on the metal sensor wires in the test cell. One Multiplate test cell incorporates two independent sensor units, each consisting of two silver-coated highly conductive wires. When the activated platelets adhere onto the sensor wires, the electrical resistance between the wires rises, which is continuously registered and transformed into the arbitrary aggregation units (AU). The area under the aggregation curve (AUC) is an estimator of the platelet aggregation expressed in arbitrary units (10 AU × min = 1 U) [25]. The definitions of both low platelet reactivity (LPR; ADP-PA < 19 U) and high platelet reactivity (HPR; ADP-PA > 46 U) were based on the consensus document of the Working Group on Thrombosis of the European Society of Cardiology [26].

Genotyping

Genomic DNA was extracted from blood according to the standard procedures. CYP2C19*2 (CYP2C19 681 G>A; rs4244285) was genotyped with a real-time allelic discrimination assay on an ABI Prism Sequence Detector 7000 (Applied Biosystems) according to the standard procedures. CYP2C19*17 (CYP2C19 806 C>T, rs12248560) was genotyped with a commercially available validated drug metabolism genotyping assay (TaqMan Drug Metabolism Genotyping Assay C_469857_10, Applied Biosystems, Foster City, CA, USA) with the ABI Prism Sequence Detector 7000 (Applied Biosystems) in accordance with the manufacturer's instructions [12]. SNPs (Single Nucleotide Polymorphisms) in ABCB1 (rs1045542) were genotyped using commercial TaqMan SNP Genotyping Assays (assay IDs: rs1045642: C_7586657_20) on a ViiA 7 Real-Time PCR (Polymerase Chain Reaction) System (Life Technologies) following the manufacturer's instructions. The proper assessment of genotypes was evaluated in a random sequencing of PCR products using a BigDye Terminator v. 3.1 sequencing kit and a 3130xl Genetic Analyzer (Applied Biosystems) [12]. The compatibility between the results of real-time allelic discrimination and direct sequencing was confirmed.

Statistical analysis

The use of the Shapiro-Wilk test demonstrated that the investigated continuous variables were not normally distributed. Therefore, continuous results were reported throughout the manuscript as median values and interquartile ranges. Categorical variables were compared using the χ^2 test with Yates's correction if needed. The correlations were tested with the Spearman's rank correlation test. The impact of the numerous variables on a quantitative variable was assessed using the multiple regression model. A two-sided difference was considered significant at $p < 0.05$. The statistical analysis was carried out using the Statistica 10.0 package (Stat-Soft, Tulsa, OK, USA).

Results

A total of 157 patients were included in the study. The characteristics of the study population are provided in Table 1. The attendance rate at the 3-month follow-up visit was 100%.

ADP-PA during the index hospitalization was significantly higher than at the 3-month follow-up visit regardless of the genotype (Tab. 2). Due to the small number of CYP2C19*2 681 G>A homozygotes, all carriers of the mutant allele were combined in one group consisting of homozygotes AA (n = 1), and heterozygotes GA (n = 26) were compared with GG homozygotes (n = 130). There were no significant differences between ADP-PA during the index hospitalization and after three months of clopidogrel therapy for loss-of-function allele of CYP2C19*2 (GA and GA+AA genotypes) (Tab. 2). We did not find any significant differences between ADP-PA both during the acute phase of myocardial infarction and after 3 months in TT homozygotes and CT heterozygotes for the C3435T variant of the ABCB1 gene (Tab. 3). Moreover, we observed higher ADP-PA in CYP2C19*17/*17 (TT homozygotes) allele carriers comparing with carriers of wild allele, but only during the index hospitalization (Tab. 2, Fig. 1). To allow the impact assessment of both of these polymorphisms (*2 loss-of-function and *17 gain-of-function) on the individual variation of clopidogrel antiplatelet action, patients were classified into extensive metabolizer, normal metabolizer and poor metabolizer groups, according to the expected metabolic activity of the CYP2C19 enzyme. There were no significant differences between ADP-PA during the index hospitalization and after three months of clopidogrel therapy for extensive metabolizers, normal metabolizers and poor metabolizers (Tab. 3). We also compared the incidence of HPR and LPR based on the genotype. There were no significant differences in the distribution of patients with LPR, optimal platelet reactivity (OPR) and HPR (Tab. 4).

In the absence of the significant association of the platelet aggregation with selected genetic polymorphisms CYP2C19*2 and ABCB1 as well as the higher value of platelet aggregation in patients with ultrafast metabolism of clopidogrel CYP2C19*17/*17, it was verified which of the selected variables influence ADP-PA

Table 1. Characteristics of the study population (n = 157). Continuous variables are presented as median (lower quartile–upper quartile), while categorical variables are presented as number (percent)

Variable	Value (%)
Age (years)	60.0 (53.0–66.0)
Gender (male/female)	115/42 (73.2/26.8)
NSTEMI	22 (14)
STEMI	135 (86)
Prior diagnosis of CAD	35 (22.3)
Prior MI	13 (8.3)
Prior PCI	12 (7.6)
Prior CABG	4 (2.5)
LVEF (%)	45 (41.0–50.0)
Heart failure on admission with NYHA class II	10 (6.4)
Heart failure at discharge with NYHA class II–IV	37 (23.6)
BMI [kg/m^2]	27.9 (25.2–30.8)
Waist circumference [cm]	96.5 (90.0–103.5)
Hyperlipidemia	98 (62.4)
Hypertension	86 (54.8)
Diabetes mellitus	54 (34.4)
Current smoker	81 (51.6)
Glycemia on admission [mg/dL]	138.0 (117.0–164.0)
WBC [10^3/μL]	7.58 (6.34–9.17)
RBC [10^6/μL]	4.52 (4.2–4.84)
HGB [g/dL]	13.5 (12.8–14.4)
HCT (%)	39.4 (37.0–42.2)
PLT [10^3/μL]	208.0 (176.0–241.0)
MPV [fL]	10.9 (10.3–11.4)
BNP [pg/dL]	105.2 (60.0–209.1)
hsCRP [mg/L]	9.43 (4.53–29.3)

BMI — body mass index; BNP — B-type natriuretic peptide; CABG — coronary artery bypass grafting; CAD — coronary artery disease; HCT — hematocrit; HGB — hemoglobin; hsCRP — high sensitivity C-reactive protein; LVEF — left ventricular ejection fraction; NYHA — New York Heart Association; MI — myocardial infarction; MPV — mean platelet volume; NSTEMI — non ST-segment elevation myocardial infarction; PCI — percutaneous coronary intervention; PLT — platelets; RBC — red blood cells; STEMI — ST-segment elevation myocardial infarction; WBC — white blood cells

during hospitalization and after three months of clopidogrel therapy.

As indicated with univariate analysis, the mean corpuscular volume of erythrocytes, mean platelet volume, white blood cell count, platelet count, diabetes, heart failure at discharge and presence of CYP2C19*17/*17 influenced platelet reactivity during the hospitalization (Tab. 5, 6). The Spearman's rank correlation test showed significant linear correlations between the value of ADP-PA measured after three months for the following variables: age, platelet count and white blood cell count (Tab. 5). Univariate analysis after three months showed the influence of heart failure at discharge on the platelet aggregation (Tab. 6).

To further investigate the relationship between ADP-PA and both, quantitative and qualitative, variables during the hospitalization and after three months of clopidogrel therapy, we applied multivariate regression analysis (Tab. 7). Mean platelet volume, platelet count and heart failure at discharge class II–IV NYHA were independently associated with an increase in ADP-PA during the hospitalization. Age, white blood cells count, platelets count and mean platelet volume were independently associated with ADP-PA after three months of clopidogrel therapy (Tab. 7). The coefficient of multiple determination R^2 was 0.278. This model explains 27.8% of the variability of ADP-PA during the hospitalization. The statistical significance of the model is $p < 0.000001$. After three months, the coefficient of multiple determination R^2 was 0.223, so this model explains 22.3% of the variability of ADP-PA after three months. The statistical significance of the model is $p < 0.000001$ (Tab. 7).

Discussion

The main finding of our study is that on-clopidogrel platelet reactivity significantly decreases beyond the acute phase of myocardial infarction regardless of the genotype. Additionally, our study indicates that in clopidogrel-treated myocardial infarction patients genetic polymorphisms are not major determinants of the interindividual variability in platelet reactivity.

We found significant reduction of ADP-PA after three months of clopidogrel therapy, independently of the presence of any genetic polymorphisms.

In a study by Campo et al., both on-clopidogrel platelet reactivity with the VerifyNow P2Y12 test and genetic polymorphisms: CYP2C19*2, *17, CYP3A5*3, and ABCB1 were evaluated in 300 patients before PCI intervention, one and six months thereafter. On-clopidogrel platelet reactivity showed a significant reduction from the index hospitalization to one month. The percentage of poor responders decreased from 35% (95% CI: 30% to 41%) at baseline to 13% (95% CI: 9% to 18%) at one month. A significant decrease of on-clopidogrel platelet reactivity was observed from baseline (index hospitalization) to one month, without further changes up to six months. This trend was more pronounced in patients admitted for non ST-segment elevation acute coronary syndrome, a consistent platelet reactivity

Table 2. Results of ADP-PA during hospitalization and after three months of clopidogrel therapy for CYP2C19*2 681 G>A, CYP2C19*17 806 C>T and ABCB1 3435 C>T genotypes, median (lower quartile–upper quartile)

CYP2C19*2 681 G>A	**GG n = 130**	**GA n = 26**	**GA + AA* n = 27**	**p value for the comparison of ADP-PA between genotypes**
ADP-PA — 10:00 a.m. hospitalization [U]	24.0 (16.0–36.0)	30.0 (16.0–36.0)	30.0 (15.0–36.0)	0.766
ADP-PA — 3 months [U]	15.5 (10.0–24.0)	18.5 (14.0–27.0)	18.0 (13.0–27.0)	0.663
p value for the comparison of ADP-PA between index hospitalization and after 3 months of clopidogrel therapy	0.00000044	0.095	0.080	
CYP2C19*17 806 C>T	**CC n = 85**	**CT n = 59**	**TT n = 13**	**p value for the comparison of ADP-PA between genotypes**
ADP-PA – 10:00 a.m. hospitalization [U]	20.0 (14.0–35.0)	25.0 (16.0–33.0)	35.0 (24.0–44.0)	0.026
ADP-PA – 3 months [U]	17.0 (11.0–26.0)	15.0 (10.0–21.0)	22.0 (16.0–30.0)	0.115
p value for the comparison of ADP-PA between index hospitalization and after 3 months of clopidogrel therapy	0.003	0.0002	0.02	
ABCB1 3435C>T	**CC n = 37**	**CT n = 79**	**TT n = 41**	**p value for the comparison of ADP-PA between genotypes**
ADP-PA — 10:00 a.m. hospitalization [U]	27.0 (15.0–40.0)	24.0 (17.0–37.0)	20.0 (13.0–28.0)	0.114
ADP-PA — 3 months [U]	15.0 (8.0–23.0)	17.0 (11.0–26.0)	17.0 (12.0–25.0)	0.327
p value for the comparison of ADP-PA between index hospitalization and after 3 months of clopidogrel therapy	0.000131	0.000343	0.098	

*Only one patient with genotype AA was identified — no statistical analysis was performed. ADP-PA — adenosine diphosphate induced platelet aggregation

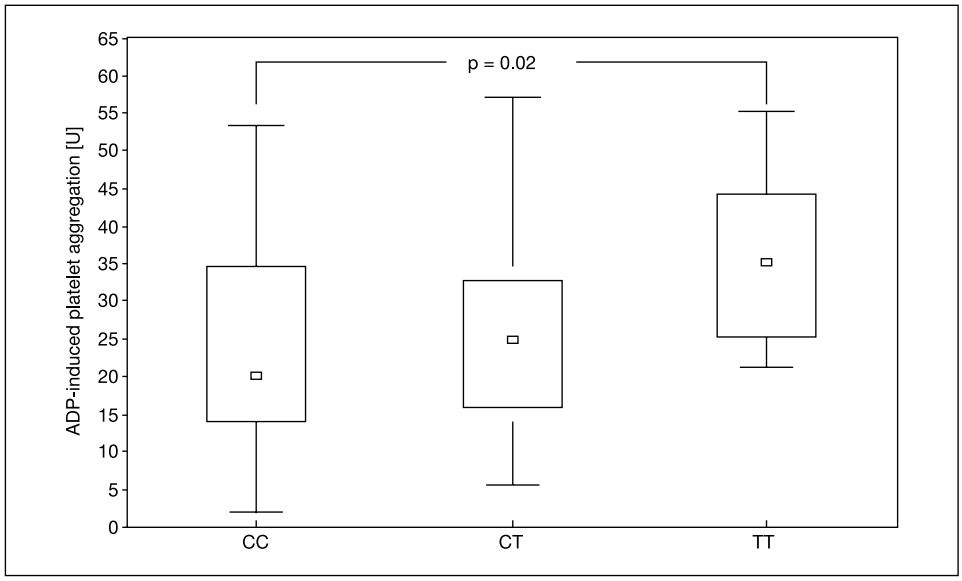

Figure 1. Comparison of ADP-PA during hospitalization in relation to the presence of CYP2C19*17 806 C>T genotypes (marker — median, the edges of the box — the upper and lower quartile)

Table 3. Results of ADP-PA during hospitalization and after 3 months of clopidogrel therapy, depending on the metabolic phenotype of clopidogrel, median (lower quartile–upper quartile)

Phenotype	Extensive metabolizers (681 GG + 806 CT or TT)	Normal metabolizers (681 GG + 806 CC and 681 GA or AA + 806 CT or TT)	Poor metabolizers (681 GA or AA + 806 CC)	p value
	n = 61	n = 80	n = 16	
ADP-PA — 10:00 a.m. hospitalization [U]	27.0 (18.0–40.0)	20.5 (14.0–32.5)	31.5 (12.5–39.0)	0.2320
ADP-PA — 3 months [U]	16.0 (10.0–23.0)	16.0 (11.0–25.0)	22.5 (14.5–28.0)	0.3727

ADP-PA — adenosine diphosphate induced platelet aggregation

Table 4. Comparison of distribution of low, optimal and high platelet reactivity during hospitalization between genotypes [n (%)]

CYP2C19*2	LPR < 19 U (n = 54)	OPR 19–46 U (n = 84)	HPR > 46 U (n = 19)
GG	45 (83.33)	68 (80.95)	17 (89.47)
GA + AA	9 (16.67)	16 (19.05)	2 (10.53)
p value		0.66817	
CYP2C19*17	**LPR < 19 U (n = 54)**	**OPR 19–46 U (n = 84)**	**HPR > 46 U (n = 19)**
CC	35 (64.81)	41 (48.81)	9 (47.37)
CT	19 (35.19)	33 (39.29)	7 (36.84)
TT	0 (0.00)	10 (11.90)	3 (15.79)
p value		0.06144	
ABCB1	**LPR < 19 U (n = 54)**	**OPR 19–46 U (n = 84)**	**HPR > 46 U (n = 19)**
CC	11 (20.37)	20 (23.81)	6 (31.58)
CT	26 (48.15)	43 (51.19)	10 (52.63)
TT	17 (31.48)	21 (25.00)	3 (15.79)
p value		0.69018	

HPR — high platelet reactivity; LPR — low platelet reactivity; OPR — optimal platelet reactivity

Table 5. Correlations between ADP-PA and quantitative variables during the index hospitalization and at 3-month follow-up visit

Index hospitalization			3-month follow-up		
Quantitative variable	**R Spearman's rank correlation coefficient**	**p value**	**Quantitative variable**	**R Spearman's rank correlation coefficient**	**p value**
MCV	0.169	0.03392	PLT	0.229	0.004098
MPV	0.203	0.01090	Age	0.285	0.000304
WBC	0.234	0.00320	WBC	0.377	< 0.000011
PLT	0.265	0.00079			

MCV — mean corpuscular volume; MPV — mean platelet volume; PLT — platelet; WBC — white blood cell

modification over time was noted also in stable patients. Gene polymorphisms justified about 18% of this trend. CYP2C19*2 and *17 influence was apparently consistent over time, whereas ABCB1 showed a higher impact at baseline [27].

Our study indicates that in the clopidogrel-treated patients genetic polymorphisms are not major determinants of the interindividual variability in platelet reactivity. However, in multivariate regression analysis mean platelet volume, platelet count and heart failure

Table 6. ADP-PA values with regard to analyzed factors during hospitalization and after three months of clopidogrel therapy

Qualitative variables	ADP-PA — 10:00 a.m. hospitalization [U]	p value
Diabetes (+) n = 54	29.0 (18.0–40.0)	0.043
Diabetes (–) n = 103	22.0 (14.0–33.0)	
Heart failure at discharge class II–IV NYHA hospitalization (+) n = 37	30.0 (18.0–43.0)	0.045
Heart failure at discharge class II–IV NYHA hospitalization (–) n = 120	22.5 (14.5–33.5)	
CYP2C19*17/*17 carriers (TT homozygotes) n = 13	35.0 (24.0–44.0)	0.010
CYP2C19*17/*17 noncarriers n = 144	23.0 (14.5–34.5)	
Qualitative variables	**ADP-PA — after 3 months of clopidogrel therapy [U]**	**p value**
Heart failure at discharge class II-IV NYHA hospitalization (+) n = 37	20.0 (14.0–31.0)	0.0087
Heart failure at discharge class II-IV NYHA hospitalization (–) n = 120	15.0 (10.0–23.5)	

ADP-PA — adenosine diphosphate induced platelet aggregation; NYHA — New York Heart Association

Table 7. Impact of clinical and biochemical variables on ADP-PA during index hospitalization and at 3-month follow-up visit

Model characteristics: $R^2 = 0.278$; $p < 0.000001$		
Variables during hospitalization	**Beta coefficient ± standard error**	**p value**
MPV	7.2271 ± 1.42020	< 0.00001
Heart failure at discharge class II–IV NYHA hospitalization	6.9414 ± 2.80661	0.014
PLT	0.1928 ± 0.02976	< 0.00001
Model characteristics: $R^2 = 0.223$; $p < 0.000001$		
Variables after 3 months of clopidogrel therapy	**Beta coefficient ± standard error**	**p value**
MPV	3.3490 ± 1.31716	0.012
WBC	1.4490 ± 0.61572	0.020
Age	0.3562 ± 0.1084	0.0013
PLT	0.1013 ± 0.025	0.00008

MCV — mean corpuscular volume; MPV — mean platelet volume; NYHA — New York Heart Association; PLT — platelets; WBC — white blood cells

at discharge are independent factors associated with ADP-PA during the hospitalization, while mean platelet volume, platelet count, white blood cell count and age are associated with ADP-PA after three months of clopidogrel therapy.

There are many variables conditioning the on-clopidogrel platelet reactivity. Undoubtedly, genetic determinants can exert influence on the clopidogrel metabolism. The polymorphisms of genes encoding the hepatic cytochrome enzymes and P-glycoprotein are acknowledged major factors of the interindividual clopidogrel response. There are many combinations of different CYP2C19 alleles impairing or enhancing activity of this enzyme [28]. It is of high importance, especially in patients after ACS, because a decreased response to antiplatelet therapy in this group may lead to atherothrombotic complications including myocardial infarction, stent thrombosis, distal thromboembolism or stroke [8]. The prevalence of alleles differs widely between populations. It has been shown that as many as about 25% of the Polish population are CYP2C19*2 heterozygotes, while about 2% are CYP2C19 2/*2 homozygotes [29]. The CYP2C19*17 allele is pretty common and accounts for 43% of the Polish population [29]. This data is consistent with our observations (Tab. 8).

The presence of CYP2C19*2 allele in healthy volunteers was associated with a significantly lower inhibition of platelet reactivity comparing to carriers of wild allele [19]. In the study by Collet et al., they found a strong correlation between the presence of CYP2C19*2 and recurrent coronary events in patients after myocardial infarction

Table 8. Genotype distribution in the study population [number (percent)]

Genotypes	**n = 157**
CYP2C19*2 681 G>A	**n (%)**
GG	130 (82.8)
GA	26 (16.6)
AA	1 (0.6)
CYP2C19*17 806 C>T	**n (%)**
CC	85 (54.1)
CT	59 (37.6)
TT	13 (8.3)
ABCB1 3435C>T	**n (%)**
CC	37 (23.6)
CT	79 (50.3)
TT	41 (26.1)

on clopidogrel therapy [30]. The primary endpoint composed of death, myocardial infarction and urgent revascularization occurred significantly more often in carriers of mutant allele (HR [hazard ratio] 3.69; 95% CI [confidence interval] 1.69–8.05; p = 0.0005), moreover, the frequency of stent thrombosis was also higher in this group (HR 6.02; 95% CI 1.81–20.04; p = 0.0009). Multivariable analysis showed the presence of CYP2C19*2 to be a single independent predictor of cardiovascular events (HR 4.04; 95% CI 1.81–9.02; p = 0.0006). Similarly, Sibbing and co-workers found a threefold higher occurrence of stent thrombosis in the presence of CYP2C19*2 mutation as compared with the wild type homozygous patients (p = 0.007) [31]. The researchers of the genetic substudy of TRITON-TIMI 38 trial (Trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel TIMI 38) collected genetic data of almost 1500 patients with ACS treated with either prasugrel or clopidogrel [32]. Over 27% of them were the carriers of mutant allele and presented with the significantly lower concentrations of clopidogrel active metabolite comparing with non-carriers, they were at the increased risk of the primary end-point composed of death from cardiovascular causes, myocardial infarction and stroke (HR 1.53; 95% CI 1.07–2.19; p = 0.01). On the other hand, the results of the CHARISMA (Clopidogrel for high atherothrombotic risk and ischemic stabilization, management and avoidance) genetics study performed in a group of 4819 patients with stable coronary artery disease revealed that the presence of CYP2C19*2 allele as well as CYP2C19*17 allele did not influence the rate of ischemic events [33]. Similar observations were made by Zabalza et al. In their meta-analysis, they showed that carriers of loss of function allele did not have an increased risk of cardiovascular complications, except for stent thrombosis, while carriers of gain of function allele had a lower risk of cardiovascular events, but a higher risk of bleeding complications [34]. In a pharmacodynamic study, Frere et al. found that the CYP2C19*17 carriers exhibit the lowest level of phosphorylation of the intracellular vasodilator stimulated phosphoprotein in flow cytometry analysis, which means that the platelet inhibition after a 600 mg loading dose of clopidogrel was the greatest in this subset of patients [18]. Also in a study by Sibbing and colleagues they assessed ADP-PA in CYP2C19*17 carriers, and found a significantly reduced platelet reactivity, which was associated with an increased bleeding risk in this group comparing to wild-type homozygotes [35].

The polymorphism of gene ABCB1 encoding P-glycoprotein, which regulates the transmembrane transport of clopidogrel, may result in an increased risk of cardiovascular events. Simon et al. found that carriers of two variant alleles 3435 C>T had a higher rate of atherothrombotic complications including myocardial infarction, death from any cause and non-fatal stroke, comparing to carriers of wild allele in the population of patients with myocardial infarction [14]. In the above mentioned genetic substudy of TRITON-TIMI, 38 trial carriers of two variant alleles 3435 C>T treated with clopidogrel had a higher platelet reactivity and were at increased risk of recurrent ischemic events [22]. Our observations did not confirm that the presence of CYP2C19*2 allele or ABCB13435 C>T was associated with a significantly lower inhibition of platelet reactivity comparing to carriers of wild allele, and the results did not confirm the increased ADP-PA in patients presenting with polymorphisms of CYP2C19*17. However, some reports could not confirm the impact of CYP2C19*2 on clinical outcome [12, 33]. This discrepancy might be explained by the fact that the CYP2C19*2 allele accounts only for 5–12 % of the variation in the response to clopidogrel [36, 37]. Thus, available data suggest that other variables like unknown genetic variants or other not identified factors contribute to this phenomenon.

Generally, according to a study by Hoshino et al., the level of platelet inhibition during clopidogrel therapy is constant in stable conditions [38]. It has been shown that mean platelet volume (MPV), is a marker of platelet reactivity [39]. An increased MPV is associated with myocardial damage in ACS and with unfavorable outcome among survivors of myocardial infarction [40, 41]. Chu et al. observed a decrease in MPV in subjects with ACS [42]. Nevertheless, higher platelet reactivity in the acute phase of myocardial infarction may be associated with enhanced platelet turnover [43]. Another import-

ant factor influencing platelet aggregation is platelet count. Wu et al., in a meta-analysis comprising a total of 39,324 patients with ACS, demonstrated that a higher platelet count enhances the risk of mortality and major cardiac adverse events [44].

Furthermore, activated platelets take part in mediation of the inflammatory response interacting with leukocytes and producing cytokines in the acute phase of ACS [45]. Inflammation, as assessed by the white blood cell count, is a well-documented risk factor of a decreased platelet response to clopidogrel [46, 47]. In a study by Osmancik et al. they found higher white blood cell count and interleukin-10 concentration as determinants for non-responsiveness to clopidogrel [48].

Cuisset et al. found significantly higher platelet reactivity in response to clopidogrel in older patients (> 75 years of age) with non-ST-elevation ACS [49]. According to multivariate analysis performed after three months of clopidogrel therapy, in our population age is also an important factor affecting the on-treatment platelet function.

Our findings suggest that heart failure at discharge (NYHA II–IV) is a clinical factor influencing the antiplatelet effect of clopidogrel, according to the multivariate analysis in the index hospitalization. Patients with heart failure may be predisposed to a hypercoagulable or prothrombotic state [50, 51]. In patients with heart failure activation of the sympathetic nervous system is associated with activation of the platelet function [52].

Limitations of the study

Several limitations of our study should be acknowledged. Firstly, we applied only one method of the platelet function assessment. Secondly, due to a limited sample size, we were not able to evaluate the impact of the investigated polymorphisms on clinical outcomes. Thirdly, we cannot exclude the minor contribution of the analyzed genetic variants to the interindividual variability of on-clopidogrel platelet reactivity. Fourthly, participants' adherence to clopidogrel therapy during the 3-month follow-up was not objectively proved. However, all patients declared systematic intake of clopidogrel. Finally, we cannot exclude the influence of other variables, which were not included in the analysis, on ADP-PA.

Conclusions

On-clopidogrel platelet reactivity significantly decreases beyond the acute phase of myocardial infarction regardless of the genotype. Additionally, our study indicates that in the clopidogrel-treated myocardial infarction patients genetic polymorphisms are not major determinants of the interindividual variability in platelet reactivity. However, due to a limited sample size, their minor contribution cannot be excluded.

References

1. CAPRIE Steering Committee. A randomized, blinded, trial of Clopidogrel Versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE). Lancet 1996; 348: 1329–1339.
2. Yusuf S, Zhao F, Mehta SR et al. Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001; 345: 494–502.
3. Sabatine MS, Cannon CP, Gibson CM et al. Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY)-Thrombolysis in Myocardial Infarction (TIMI) 28 Investigators. Effect of clopidogrel pretreatment before percutaneous coronary intervention in patients with ST-elevation myocardial infarction treated with fibrinolytics: the PCI-CLARITY study. JAMA 2005; 294: 1224–1232.
4. Windecker S, Kolh P, Alfonso F et al. 2014 ESC/EACTS Guidelines on myocardial revascularization. The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS). Eur Heart J 2014; 35: 2541–2619.
5. Kozinski M, Bielis L, Wisniewska-Szmyt J et al. Diurnal variation in platelet inhibition by clopidogrel. Platelets 2011; 22: 579–587.
6. Gurbel PA, Bliden KP, Guyer K et al. Platelet reactivity in patients and recurrent events post–stenting: Results of the PREPARE POST-STENTING Study. J Am Coll Cardiol 2005; 46: 1820–1826.
7. Buonamici P, Marcucci R, Migliorini A et al. Impact of platelet reactivity after clopidogrel administration on drugeluting stent thrombosis. J Am Coll Cardiol 2007; 49: 2312–2317.
8. Matetzky S, Shenkman B, Guetta V et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation 2004; 109: 3171–3175.
9. Barsky AA, Arora RR. Clopidogrel resistance: myth or reality? J Cardiovasc Pharmacol Ther 2006; 11: 47–53.
10. Kubica A, Koziński M, Grześk G, Goch A. Clinical significance of interactions between clopidogrel and proton pump inhibitors. Kardiol Pol 2011; 69: 610–616.
11. Pereillo JM, Maftouh M, Andrieu A et al. Structure and stereochemistry of the active metabolite of clopidogrel. Drug Metab Dispos 2002; 30: 1288–1295.
12. Siller-Matula JM, Delle-Karth G, Lang IM et al. Phenotyping vs. genotyping for prediction of clopidogrel efficacy and safety: the PEGASUS--PCI study. J Thromb Haemost 2012; 10: 529–542.
13. Navarese EP, Verdoia M, Schaffer A et al. Ischaemic and bleeding complications with new, compared to standard, ADP-antagonist regimens in acute coronary syndromes: a meta–analysis of randomized trials. QJM 2011; 104: 561–569.
14. Simon T, Verstuyft C, Mary-Krause M et al. French Registry of Acute ST–Elevation and Non-ST-Elevation Myocardial Infarction (FAST-MI) Investigators: Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 2009; 360: 363–375.
15. Trenk D, Hochholzer W, Fromm MF et al. Cytochrome P450 2C19 681G_A polymorphism and high on-clopidogrel platelet reactivity associated with adverse 1-year clinical outcome of elective percutaneous coronary intervention with drug-eluting or bare-metal stents. J Am Coll Cardiol 2008; 51: 1925–1934.
16. Hulot JS, Bura A, Villard E et al. Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood 2006; 108: 2244–2247.
17. Storey RF. Clopidogrel in acute coronary syndrome: to genotype or not? Lancet 2009; 373: 276–278.
18. Frére C, Cuisset T, Gaborit B et al. The CYP2C19*17 allele is associated with better platelet response to clopidogrel in patients admitted for non--ST acute coronary syndrome. J Thromb Haemost 2009; 7: 1409–1411.
19. Hulot JS, Collet JP, Silvain J et al. Cardiovascular risk in clopidogrel-treated patients according to cytochrome P450 2C19*2 loss-of-function allele or proton pump inhibitor co-administration. A systematic meta--analysis. J Am Coll Cardiol 2010; 56: 134–143.
20. Varenhorst C, James S, Erlinge D et al. Genetic variation of CYP2C19 affects both pharmacokinetic and pharmacodynamic responses to

clopidogrel but not prasugrel in aspirin-treated patients with coronary artery disease. Eur Heart J 2009; 30: 1744–1752.
21. Taubert D, von Beckerath N, Grimberg G et al. Impact of P-glycoprotein on clopidogrel absorption. Clin Pharmacol Ther 2006; 80: 486–501.
22. Mega JL, Close SL, Wiviott SD et al. Genetic variants in ABCB1 and CYP2C19 and cardiovascular outcomes after treatment with clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis. Lancet 2010; 376: 1312–1319.
23. Gremmel T, Panzer S. Clinical, genetic and confounding factors determine the dynamics of the in vitro response/non response to clopidogrel. Thromb Haemost 2011; 106: 211–218.
24. Marcucci R, Gori AM, Paniccia R et al. Residual platelet reactivity is associated with clinical and laboratory characteristics in patients with ischemic heart disease undergoing PCI on dual antiplatelet therapy. Atherosclerosis 2007; 195: 217–223.
25. Cattaneo M. Resistance to antiplatelet drugs: molecular mechanisms and laboratory detection. J Thromb Haemost 2007; 5 (suppl. 1): 230–237.
26. Aradi D, Storey RF, Komócsi A et al. Working Group on Thrombosis of the European Society of Cardiology. Expert position paper on the role of platelet function testing in patients undergoing percutaneous coronary intervention. Eur Heart J 2014; 35: 209–215.
27. Campo G, Parrinello G, Ferraresi P et al. Prospective evaluation of on–clopidogrel platelet reactivity over time in patients treated with percutaneous coronary intervention relationship with gene polymorphisms and clinical outcome. J Am Coll Cardiol 2011; 57: 2474–2483.
28. Maeda A, Ando H, Asai T et al. Differential impacts of CYP2C19 gene polymorphisms on the antiplatelet effects of clopidogrel and ticlopidine. Clin Pharmacol Ther 2011; 89: 229–233.
29. Kubica A, Kozinski M, Grzesk G et al. Genetic determinants of platelet response to clopidogrel. J Thromb Thrombolysis 2011; 32: 459–466.
30. Collet JP, Hulot JS, Pena A et al. Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. Lancet 2009; 373: 309–317.
31. Sibbing D, Stegherr J, Latz W et al. Cytochrome P450 2C19 loss-of-function polymorphism and stent thrombosis following percutaneous coronary intervention. Eur Heart J 2009; 30: 916–922.
32. Mega JL, Close SL, Wiviott SD et al. Cytochrome P-450 polymorphisms and response to clopidogrel. N Engl J Med 2009; 360: 354–362.
33. Bhatt DL, Paré G, Eikelboom JW et al. The relationship between CYP2C19 polymorphisms and ischaemic and bleeding outcomes in stable outpatients: the CHARISMA genetics study. Eur Heart J 2012; 33: 2143–2150.
34. Zabalza M, Subirana I, Sala J et al. Meta-analyses of the association between cytochrome CYP2C19 loss- and gain-of-function polymorphisms and cardiovascular outcomes in patients with coronary artery disease treated with clopidogrel. Heart 2012; 98: 100–108.
35. Sibbing D, Koch W, Gebhard D et al. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation 2010; 121: 512–518.
36. Shuldiner AR, O'Connell JR, Bliden KP et al. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 2009; 302: 849–857.
37. Hochholzer W, Trenk D, Fromm MF et al. Impact of cytochrome P450 2C19 loss-of-function polymorphism and of major demographic characteristics on residual platelet function after loading and maintenance treatment with clopidogrel in patients undergoing elective coronary stent placement. J Am Coll Cardiol 2010; 55: 2427–2434.
38. Hoshino K, Horiuchi H, Tada T et al. Clopidogrel resistance in Japanese patients scheduled for percutaneous coronary intervention. Circ J 2009; 73: 336–342.
39. Khode V, Sindhur J, Kanbur D et al. Mean platelet volume and other platelet volume indices in patients with stable coronary artery disease and acute myocardial infarction: A case control study. J. Cardiovasc Di. Res 2012; 3: 272–275.
40. Kiliçli-Camur N, Demirtunç R, Konuralp C et al. Could mean platelet volume be a predictive marker for acute myocardial infarction? Med Sci Monit 2005; 11: 387–392.
41. Endler G, Klimesch A, Sunder–Plassmann H et al. Mean platelet volume is an independent risk factor for myocardial infarction but not for coronary artery disease. Br J Haematol 2002; 117: 399–404.
42. Chu H, Chen WL, Huang CC et al. Diagnostic performance of mean platelet volume for patients with acute coronary syndrome visiting an emergency department with acute chest pain: The Chinese scenario. Emerg Med J 2011; 28: 569–574.
43. Funck–Jensen KL, Dalsgaard J, Grove EL et al. Increased platelet aggregation and turnover in the acute phase of ST-elevation myocardial infarction. Platelets 2014; 24: 528–537.
44. Wu Y, Wu H, Mueller C et al. Base-line platelet count and clinical outcome in acute coronary syndrome. Circ J 2012; 76: 704–711.
45. Yokoyama S, Ikeda H, Haramaki N et al. Platelet P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet–leukocyte aggregates. J Am Coll Cardiol 2005; 45: 1280–1286.
46. Aksu K, Donmez A, Keser G. Inflammation-induced thrombosis: mechanisms, disease associations and management. Curr Pharm Des 2012; 18: 1478–1493.
47. Morel O, El Ghannudi S, Hess S et al. The extent of P2Y12 inhibition by clopidogrel in diabetes mellitus patients with acute coronary syndrome is not related to glycaemic control: roles of white blood cell count and body weight. Thromb Haemost 2012; 108: 338–348.
48. Osmancik P, Paulu P, Tousek P et al. High leukocyte count and interleukin-10 predict high on–treatment platelet reactivity in patients treated with clopidogrel. J Thromb Thrombolysis 2012; 33: 349–354.
49. Cuisset T, Quilici J, Grosdidier C et al. Comparison of platelet reactivity and clopidogrel response in patients ≤ 75 Years Versus > 75 years undergoing percutaneous coronary intervention for non-ST--segment elevation acute coronary syndrome. Am J Cardiol 2011; 108: 1411–1416.
50. Yamamoto K, Ikeda U, Furuhashi K et al. The coagulation system is activated in idiopathic cardiomyopathy. J Am Coll Cardiol 1995; 25: 1634–1640.
51. Mehta J, Mehta PI. Platelet function studies in heart disease. VI. Enhanced platelet aggregate formation activity in congestive heart failure: inhibition by sodium nitroprusside. Circulation 1979; 60: 497–503.
52. Jafri SM, Ozawa T, Mammen E, et al. Platelet function, thrombin and fibrinolytic activity in patients with heart failure. Eur Heart J 1993; 14: 205–212.