European Review for Medical and Pharmacological Sciences 2021; 25: 6065-6076

The correlation between platelet responsiveness to clopidogrel and CYP2C19 polymorphism in patients with peripheral vascular disease

N.M. EL-KHODARY[1,6], A.M. EL-BEHERY[1], N.A. EL-ASKARY[2], H.M. DONIA[3], G.A. OMRAN[4]

[1]Clinical Pharmacy and Pharmacy Practice Department, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt
[2]Vascular Surgery Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt
[3]Clinical and Chemical Pathology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt
[4]Biochemistry Department, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt

Abstract. – OBJECTIVE: Several patients undergoing endovascular intervention and bypass surgery present with high platelet reactivity following clopidogrel treatment. We aimed to determine the frequency of the genetic polymorphism of *CYP2C19*2* and the contribution of this polymorphism along with other clinical parameters to clopidogrel response in an Egyptian population.

PATIENTS AND METHODS: A total of 50 patients receiving clopidogrel at a maintenance dose of 75 mg daily post vascular intervention from January 1, 2019, to May 30, 2020, were enrolled in this study. Clopidogrel resistance was determined through platelet aggregation analysis using Chrono-Log® platelet aggregometer. Single-nucleotide polymorphism (SNP) genotyping was performed using quantitative real-time polymerase chain reaction (QRT-PCR).

RESULTS: The incidence of clopidogrel resistance among this Egyptian population is about 22%. Univariate analysis demonstrated that *CYP2C19*2* genotype ($p = 0.001$), high body mass index (BMI; $p = 0.025$), diabetes ($p = 0.037$), high fasting blood glucose (FBG) level ($p = 0.037$), and high glycosylated hemoglobin (HbA1c) level ($p = 0.004$) were significantly associated with clopidogrel resistance. Multivariate analysis showed that *CYP2C19*2* genotype (odds ratio (OR), 927.71; 95% confidence interval (CI), 1.915–449496.2; $p = 0.030$) and high BMI (OR, 1.789; 95% CI, 1.044–3.064; $p = 0.034$) were the most powerful predictors of clopidogrel resistance.

CONCLUSIONS: Clopidogrel resistance in patients with peripheral vascular disease is associated with the presence of *CYP2C19*2* allele, obesity, and diabetes; these factors should be considered prior to clopidogrel administration.

Key Words:
Clopidogrel resistance, Peripheral vascular disease, Risk factors, CYP2C19, High on-treatment platelet reactivity, Antiplatelet therapy.

Introduction

Lower extremity peripheral artery disease (PAD) is prevalent, affecting more than 200 million individuals worldwide. The generation of thrombi resulting from platelet activation and aggregation is the most critical step involved in atherosclerotic vascular diseases, such as PAD[1]. Patients with PAD may be asymptomatic or present with symptoms such as intermittent claudication (IC) or critical limb ischemia (CLI), in addition to rest pain and trophic changes. CLI is an absolute indication for revascularization to achieve limb salvage and avoid major amputation[1,2]. Patients with PAD bear an increased risk of mortality and presenting with cardiovascular events such as stroke and acute myocardial infarction[1]. Pharmacotherapy aims at decreasing the risk of future cardiovascular morbidity (and subsequent mortality) in patients with PAD, improving the walking ability of patients with IC, and reducing major amputation in patients with CLI. Secondary prevention is facilitated through using antiplatelet agents and modifying other risk factors such as tobacco use, hypertension, hperlipidemia, and diabetes[3].

A large-scale study showed that clopidogrel is superior to aspirin in decreasing the risk of isch-

Corresponding Author: Noha Mahmoud El-Khodary, Ph.D; e-mail: nohamahmoud_55@hotmail.com; noha.mahmoud@pharm.dmu.edu.eg

emic stroke in patients with stroke, myocardial infarction, or PVD[4]. Clopidogrel is an antiplatelet agent and a prodrug that is activated by the hepatic cytochrome P450 to give the active metabolite (thiol) that irreversibly connects to the platelet surface of the adenosine diphosphate (ADP) receptor P2Y12. While clopidogel is commonly used, a big interindividual reaction variability exists[5]. This clopidogrel responsiveness variability is believed to be multifactorial and include both extrinsic and intrinsic variables. Extrinsic variables include patient noncompliance, inappropriate dosage or underdosing, and the occurenece of drug–drug interactions. Intrinsic variables include high pretreatment levels of platelet reactivity, intestinal drug absorption variability, and the prescence of genetic polymorphism[6]. Platelets play an important role in the pathogenesis of a disease, and as a result, platelet function testing has become increasingly necessary as it helps determine drug efficacy[7]. In fact, several platelet function assays have been designed to establish an optimal therapeutic window for platelet inhibition. These assays aim to tailor antiplatelet therapies in the treatment of PAD, the use of a light transmission aggregometry (LTA) being among them[8]. The notion of clopidogrel resistance or high on-treatment platelet reactivity (HTPR) is used to define patients who, despite taking antiplatelet treatment, demonstrate higher platelet reactivity than the reference range. Therefore, those patients are at greater danger of presenting with ischemic events[9]. This concept was initially identified in patients with coronary artery disease; however, HTPR in patients with PAD (especially those undergoing percutaneous transluminal angioplasty (PTA)) has also been recently documented by a limited number of studies[9]. One should note that HTPR has been related to major adverse events (reintervention, major amputation, and cardiovascular death) during follow-up in patients with PAD[2].

CYP2C19 is the most studied enzyme in the metabolism of clopidogrel. More than 2,000 single-nucleotide polymorphisms (SNPs) have been identified in the *CYP2C19* gene, including 35 registered alleles in the Pharmacogene Variation Consortium[10]. However, only SNPs with an allele frequency above 5% (such as the *2, *3, and *17) have been more extensively studied[11]. The *CYP2C19*2* and *CYP2C19*3* alleles are associated with decreased or absent enzyme activity, whereas the *CYP2C19*17* allele is associated with ultrarapid enzyme activity[12]. *CYP2C19*2* (rs4244285, 681G>A) is the most common nonfunctional allele, with a frequency of 25%–30% in Caucasians, 30% in Africans, and 40%–50% in East Asians[13]. Hyporesponse to clopidogrel is correlated with *CYP2C19* nonfunction or low-function polymorphisms, as demonstrated by reduced active metabolite concentrations of the drug. Therefore, the risk of adverse vascular occurrences is greater in patients with such polymorphisms[14]. The Food and Drug Administration added a black box warning to clopidogrel as a result of this gene polymorphism[12]. The present study aims to determine the frequency of the genetic polymorphism of *CYP2C19*2* in an Egyptian population with PAD and assess the contribution of this polymorphism along with other clinical parameters to clopidogrel response. The obtained data may help improve individualized antiplatelet treatment options for patients with PAD and reduce adverse side effects. To our knowledge, this study is the first to evaluate the correlation between platelet responsiveness to clopidogrel and CYP2C19 polymorphism in Egyptian patients with PAD.

Patients and Methods

Study Design

This is an observational cross-sectional study that has recruited 50 patients. This study was conducted at the Alexandria Main University Hospital (AMUH) between January 2019 and May 2020. Approval was obtained from the Research Ethics Committee of the Faculty of Pharmacy of Damanhour University and from the Ethics Committee of AMUH, Egypt. The study adhered to the principles of the Declaration of Helsinki with regard to ethical principles for research involving human subjects.

Patients

The inclusion criteria for this study include Patients aged >30 years who use clopidogrel as an antiplatelet therapy based on a physician's diagnosis and who were on a maintenance dose of 75 mg for at least one week post vascular intervention. The exclusion criteria include the use of aspirin, anticoagulants, and/or fibrinolytics within 30 days prior to testing; the concomitant use of drugs affecting clopidogrel metabolism, such as proton pump inhibitors, calcium channel blockers, and/or statins (except pravastatin); the concomitant use of any other ADP antagonists; the presence of an active neoplasm or a history of

neoplasm; a family history of bleeding disorders; severe hepatic insufficiency; chronic kidney disease; and platelet count <100,000/mm^3.

Data Collection and Patient Interview

The study goals and procedures were clarified both verbally and in writing through the consent form to each chosen patient. Before attending, all patients signed the consent form. Data were collected from the medical file of patients into a data collection form; information included the gender, age, height, weight, clopidogrel use, medical history, surgical history, and lifestyle of patients, in addition to present and past medication.

Sample Collection and Handling

Blood samples (6 ml) were obtained from peripheral veins of participants using complete aseptic technique into tri-potassium ethylenediaminetetraacetic acid (K3EDTA) tubes and into 3.2% tri-sodium citrate tubes for platelet function tests. The obtained blood samples were directly sent to the hematology laboratory for platelet aggregation testing and were subsequently analyzed within a period ranging from 0.5 to 3 h using Chrono-Log® platelet aggregometer (Chrono-Log Corp., Havertown, PA, USA). The K3EDTA tube samples were then used for DNA extraction and molecular analysis.

Demographic and Biochemical Measurements

Body mass index (BMI) was calculated as (weight in kg) / (height in m)^2. Patient laboratory data on complete blood count, lipid profile (total cholesterol, TC; triglycerides, TG; high-density lipoprotein, HDL; and low-density lipoprotein, LDL), serum creatinine, fasting blood glucose (FBG), and glycosylated hemoglobin (HbA1c) were taken from the patients' medical files.

Platelet Aggregation Assay

Platelet aggregation was assessed using LTA. LTA was performed on platelet-rich plasma (PRP) using the turbidimetric method in a four-channel aggregometer (Chrono-Log 490 Model, Chrono-Log Corp., Havertown, PA, USA) according to standard protocols[15]. The PRP and the platelet-poor plasma (PPP) were separated by centrifugation. The PRP was obtained as a supernatant after the centrifugation of citrated blood at 170 g for 15 min, and the PPP was obtained following a second centrifugation of the samples at 2,400 g for 20 min. Light transmission was adjusted to 0% with PRP and to 100% with PPP for each measurement. Curves were recorded over a period of 5 min, and platelet aggregation was determined as the maximal percent change in light transmittance from baseline using PPP as a reference[15]. ADP (Bio Top Medical Company, USA) at a final concentration 10 µM was used to assess the P2Y12-dependent pathway aggregation. HPR was defined as more than or equal to 60% of the maximal ADP-induced aggregation. The increase in light transmittance is directly proportional to the amount of aggregation, and it was amplified and recorded as a signal on a chart paper or digitized into a computer using the AGGRO/LINK® Opti8™ software.

Pharmacogenetic Analysis

Genomic DNA was extracted from EDTA-anticoagulated whole blood. The obtained samples were processed using a QIAamp Genomic DNA Purification Kit # K51104 (QIAGEN, Germany) via a column method. The extracted DNA concentration and purity were evaluated according to the manufacturer's protocol using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the absorbent wavelengths from 260 to 280 nm. The extracted DNA was stored at -80°C until used[16].

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) for CYP2C19 Gene

The extracted DNAs were genotyped using validated TaqMan Master Mix and TaqMan SNP Genotyping Assay primer and probes (C_25986767_70, Applied Biosystems, Foster City, CA, USA). The SNP genotype G681A (rs4244285) was surveyed to characterize the allele *2. CYP2C19 polymorphisms were determined using QRT-PCR (Stratagene Real-Time PCR System; Applied Biosystems, USA). The final reaction volume of 25 µL consisted of 2 µL of template DNA, 9.25 µL DNase, RNase-free water, 12.5 µL of 2X TaqMan Universal Master Mix (Applied Biosystems, USA), and 1.25 µL of working stock of SNP genotyping assay, according to the manufacturer's recommendations. The amplification reaction was carried out as follows: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, and annealing/extension at 60 °C for 1 min. All experiments were conducted in duplicate[17]. Fluorescence signal intensity due to the degradation of the TaqMan probe was quantified during

the annealing/denaturation phase of each PCR cycle. For the allelic discrimination, two probes with different reporters were used: one that gives green color (FAM) and another that gives yellow color (VIC). An increase in the fluorescence signal of a particular dye indicates homozygosity for the allele whose probe was labeled by that dye, whereas an increase in both signals indicates heterozygosity for both alleles.

Follow-Up and Clinical Outcomes

Follow-up was performed at 1, 3, 6, and 12 months post vascular intervention using duplex ultrasound imaging and assessing the ankle–brachial index. During follow-up, if the treated lesions demonstrated restenosis, computed tomography angiography was used to confirm the particulars of the lesions. Clopidogrel adherence was also assessed during the examination. The endpoints of our study were death, major amputation, and limb salvage.

Statistical Analysis

Data were fed to the computer and analyzed using the IBM SPSS software package version 20.0 (IBM Corp., (Armonk, NY, USA). Qualitative data were described using number and percentage. Quantitative data were described using mean ± SD. The Kolmogorov–Smirnov test was used to verify the normality of the data distribution. The Chi-squared test was used to test SNP for Hardy–Weinberg equilibrium. Categorical variables were analyzed using the Chi-squared and Fisher's exact tests. Quantitative variables with normal distribution were compared using the Student's *t*-test, whereas quantitative variables with abnormal distribution were compared using the Mann–Whitney test. The significance of the obtained results was judged at the 5% level. Logistic regression was used to determine the predictive power of different variables.

Results

This study was conducted on 50 patients who were taking clopidogrel at a maintenance dose of 75 mg daily. Based on their platelet aggregation test result, the patients were divided into two groups: the clopidogrel-resistant group (n = 11; for those scoring more than or equal to 60%) and the clopidogrel-sensitive group (n = 39; for those scoring less than 60%). The demographic and clinical characteristics of the participants are presented in Table I. The mean age of all participants was 58.54 ± 10.30 years. The study included 38 males (76%) and 12 females (24%); of these patients, 21 have hypertension (42%), and 26 have diabetes patients (52%). Among all the participants, 21 have dyslipidemia (42%), and 24 were smokers (48%).

Table II presents the distribution of the study patients according to clinical presentation, whereas Table III presents the distribution of patients according to the type of procedure performed. The clinical characteristics and current medication use of patients both in the clopidogrel-resistant and clopidogrel-sensitive groups are summarized in Table IV. The mean age was 60.27 ± 9.05 and 58.05 ± 10.69 years for the clopidogrel-resistant and clopidogrel-sensitive groups, respectively. The

Table I. Demographics and clinical characteristics of patients participating in the study.

Variable	All patients (n = 50)
Gender (M/F)	38:12 (3.1:1)
Age (years)	58.54 ± 10.30
BMI (kg/m^2)	28.85 ± 3.93
Hypertension	21 (42.0%)
Diabetes	26 (52.0%)
Dyslipidemia	21 (42%)
Smoking	24 (48%)
Total Cholesterol (mg/dl)	179.9 ± 49.71
Triglycerides (mg/dl)	122.3 ± 57.60
HDL (mg/dl)	42.91 ± 9.69
LDL (mg/dl)	112.8 ± 50.87
Serum Creatinine (mg/dl)	1.0 ± 0.26
FBG (mg/dl)	154.0 ± 69.90
HbA1c (%)	8.04 ± 1.96
Hemoglobin (g/dl)	12.57 ± 1.84
Hematocrit (%)	36.62 ± 5.36
Platelets count (10^3/mm^3)	250.6 ± 79.84

BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin.

Table II. Distribution of patients participating in the study according to clinical presentation (n=50).

Clinical presentation	Number of patients
Intermittent claudication	10
Rest pain	15
Gangrenous toes& minor amputation stump	25

Table III. Distribution of patients participating in the study according to type of procedure (n=50).

Procedure	Number of patients	
Endovascular	15	Tibial angioplasties (PTA)
	10	Superficial femoral artery (SFA) + tibial angioplasty
	5	Iliac stenting
Open surgery	5	Aorto bifemoral bypass graft (ABF)
	6	Femoropopliteal bypass graft
	1	ABF bypass + femoropopliteal bypass
Hybrid procedure (endovascular + open surgery)	6	Femoropopliteal bypass + tibial angioplasty
	2	Iliac stenting + femoropopliteal bypass

clopidogrel-resistant group included 7 males (63.6%) and 4 females (36.4%), whereas the clopidogrel-sensitive group included 31 males (79.5%) and 8 females (20.5%). Those who were clopidogrel-resistant had significantly higher BMI than those who were clopidogrel-sensitive (31.38% *vs.* 28.13%, $p = 0.014$). Moreover, a medical history of diabetes was significantly associated with clopidogrel resistance (81.8%, $p = 0.025$). It was observed that none of the concurrent medication used was significantly associated with clopidogrel resistance, and no significant association was identified between the response to clopidogrel and gender, age, hypertension, smoking, or dyslipidemia. Among the tested biochemical and hematological parameters (TC, TG, HDL, LDL, Hb, hematocrit, platelet count, and serum creatinine), only FBG and HbA1c were associated with a decrease in clopidogrel response, with *p*-values of 0.003 and <0.001, respectively (Table V).

The genotyping and allele frequencies of rs4244285 were analyzed for all study participants. The results showed that heterozygote *GA* carriers were more resistant to clopidogrel than the wild-type patients carrying a *GG* ($p < 0.05$). Therefore, *A* allele carriers were more resistant to clopidogrel than *G* allele carriers ($p < 0.001$). The *G* allele of rs4244285 SNP was more common than *A* allele in the studied population (93% *vs*.7%; $p < 0.001$). The genotype distribution of the studied SNP was consistent with the Hardy–Weinberg equilibrium ($p > 0.05$; Table VI).

A correlation was identified between the clinical characteristics of patients (hypertension, diabetes, dyslipidemia, and smoking) and the distribution of the 681G >A SNP. All patients

Table IV. Comparison of clinical characteristics and current medication use between the clopidogrel resistant and sensitive groups.

Characteristics	Clopidogrel resistant (n = 11)	Clopidogrel sensitive (n = 39)	*p*-value
Gender (M/F)	7:4 (1.75:1)	31:8 (3.8:1)	0.424
Age (years)	60.27 ± 9.05	58.05 ± 10.69	0.533
BMI (kg/m^2)	31.38 ± 2.04	28.13 ± 4.05	0.014*
Hypertension	5 (45.5%)	16 (41%)	1.000
Diabetes	9 (81.8%)	17 (43.6%)	0.025*
Dyslipidemia	6 (54.5%)	15 (38.5%)	0.491
Smoking	6 (54.5%)	18 (46.2%)	0.623
Anti-hypertensive drugs			1.000
ACEI	2 (40.0%)	5 (31.3%)	
ARB	1 (20.0%)	4 (25.0%)	
B-blocker	2 (40.0%)	7 (43.8%)	
Anti-diabetic drugs			
Oral hypoglycemic	6 (54.5%)	8 (20.5%)	0.052
Insulin	3 (27.3%)	9 (23.1%)	1.000
Statin			0.311
Pravastatin	6 (54.5%)	14 (35.9%)	
H2RA			0.351
Ranitidine	3 (27.3%)	5 (12.8%)	

H2RA: H2-receptor antagonist; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker.

Table V. Comparison of laboratory data between the clopidogrel resistant and sensitive groups.

Lab variable	Clopidogrel resistant (n = 11)	Clopidogrel sensitive (n = 39)	*p*-value
Cholesterol (mg/dl)	203.8 ± 49.58	173.2 ± 48.24	0.061
Triglycerides (mg/dl)	149.5 ± 75.59	114.7 ± 50.01	0.186
HDL (mg/dl)	41.18 ± 9.08	43.39 ± 9.91	0.509
LDL (mg/dl)	132.7 ± 61.20	107.2 ± 46.95	0.128
Hemoglobin (g/dl)	12.27 ± 1.75	12.65 ± 1.88	0.552
Hematocrit (%)	38.74 ± 6.27	38.59 ± 5.16	0.937
Platelets count (10^3/mm^3)	265.1 ± 74.79	246.6 ± 81.68	0.502
Serum creatinine (mg/dl)	1.07 ± 0.27	0.98 ± 0.26	0.318
FBG (mg/dl)	212.9 ± 49.79	137.4 ± 66.05	0.003*
HbA1c (%)	9.88 ± 0.73	7.52 ± 1.89	< 0.001*

Data are presented as mean ± SD.

Table VI. Genotypes distribution and allele frequencies of rs4244285 polymorphism.

Genotype and alleles	Clopidogrel resistant (n = 11) n (%)	Clopidogrel sensitive (n = 39) n (%)	*p*-value
rs4244285			
GG (1*/1*)	5 (45.5%)	38 (97.4%)	< 0.001*
GA (1*/2*)	6 (54.5%)	1 (2.6%)	
AA (2*/2*)	0 (0.0%)	0 (0.0%)	
HWE	0.214	0.935	
Allele G (1*)	16 (72.7%)	77 (98.7%)	< 0.001*
Allele A (2*)	6 (27.3%)	1 (1.3%)	

CYP2C19*2: cytochrome p450; HWE: p-value for Hardy-Weinberg equilibrium.

were divided into two subgroups: (1) the wild group (n = 43) and (2) the heterozygous group (n = 7). No significant correlation between them was observed ($p > 0.05$; Table VII).

Binary logistic regression was used to determine factors associated with clopidogrel resistance in patients with PAD. The univariate logistic regression demonstrated that several risk factors, such as a *CYP2C19*2* genotype, high BMI, diabetes, and high FBG and high HbA1c levels, were significantly correlated with clopidogrel resistance ($p < 0.01$): *CYP2C19*2* genotype (odds ratio (OR), 45.60; 95% confidence interval (CI), 4.512–460.89; $p = 0.001$), high BMI (OR, 1.273; 95% CI, 1.031–1.573; $p = 0.025$), diabetes (OR, 5.824; 95% CI, 1.110–30.559; $p = 0.037$), high FBG level (OR, 1.018; 95% CI, 1.005–1.030; $p = 0.005$) and high HbA1c level (OR, 2.453; 95% CI, 1.332–4.517; $p = 0.004$). The multivariate regression analysis showed that only the *CYP2C19*2* carriage and high BMI should be considered as significantly associated with clopidogrel resistance: *CYP2C19*2* genotype (OR, 927.71; 95% CI, 1.915–449496.2; $p = 0.030$) and high BMI (OR, 1.789; 95% CI, 1.044–3.064; $p = 0.034$; Table VIII).

Table IX shows the clinical outcomes of both the clopidogrel-resistant and clopidogrel-sensitive groups during the one-year follow-up period. During the follow-up, four deaths (8%) were recorded: One in the clopidogrel-sensitive group

Table VII. Correlation between CYP2C19*2 and risk factors.

Risk factors	CYP2C19*2		*p*-value
	Wild (n = 43)	Heterozygous (n = 7)	
Hypertension	17 (39.5%)	4 (57.1%)	0.434
Diabetes	21 (48.8%)	5 (71.4%)	0.420
Dyslipidemia	16 (37.2%)	5 (71.4%)	0.115
Smoking	21 (48.8%)	3 (42.9%)	1.000

CYP2C19*2: cytochrome p450.

Table VIII. Univariate and multivariate analysis of factors associated with clopidogrel resistance in peripheral vascular disease patients.

Factors	Univariate		Multivariate	
	p-value	OR (95% CI)	*p*-value	OR (95% CI)
BMI	0.025*	1.273 (1.031-1.573)	0.034*	1.789 (1.044-3.064)
Diabetes	0.037*	5.824 (1.110-30.559)	0.136	26.264 (0.356-1937.78)
TC	0.091	1.012 (0.998-1.025)		
TG	0.088	1.010 (0.999-1.021)		
LDL	0.161	1.009 (0.997-1.021)		
FBG	0.037*	1.018 (1.005-1.030)	0.215	1.022 (0.988-1.057)
HbA1c	0.004*	2.453 (1.332-4.517)	0.097	3.291 (0.805-13.464)
CYP2C19*2	0.001*	45.60 (4.512-460.89)	0.030*	927.713 (1.915-449496.2)

BMI: body mass index; TC: total cholesterol; TG: triglycerides; LDL: low density lipoprotein; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; CYP2C19*2: cytochrome p450; OR: odds ratio; CI: confidence interval.

Table IX. Comparison between the two studied groups according to adverse events in one-year follow-up.

Adverse events	Clopidogrel resistant (n = 11)	Clopidogrel sensitive (n = 39)	*p*-value
Re-intervention	2 (18.2%)	1 (2.6%)	0.118
Major amputation	3 (27.3%)	1 (2.6%)	0.029*
Non-fatal MI	1 (9.1%)	1 (2.6%)	0.395
Minor stroke	2 (18.2%)	1 (2.6%)	0.118
Death	3 (27.3%)	1 (2.6%)	0.029*

MI: myocardial infarction.

(due to uncontrolled gastrointestinal tract bleeding at 6 months) and three in the clopidogrel-resistant group (one of them due to extensive myocardial infarction at 1 month and the other two due to massive stroke at 2 and 3 months). Among the survivors, three (6%) underwent re-intervention, four (8%) underwent major amputation, two (4%) suffered a nonfatal myocardial infarction, and three (6%) had a minor stroke. The incidences of major amputation and death in the clopidogrel-resistant group were significantly higher than those in the clopidogrel-sensitive group (27.3% *vs.* 2.6%; $p = 0.029$). In our study, the amputation-free survival rate was 84%, and the limb salvage rate was 92%.

A correlation was identified between patient clinical outcomes and the distribution of the 681G>A SNP. All patients were divided into two subgroups: (i) a wild (n = 43) and (ii) a heterozygous group (n = 7). The results showed that the incidence of major amputation in the heterozygous group was significantly higher than that of the wild group (57.1% *vs.* 0.0%; $p < 0.001$). In addition, the occurrence frequency of minor strokes in the heterozygous group was significantly higher than in the wild group (28.6% *vs.* 2.3%; $p = 0.048$; Table X).

Table X. Correlation between CYP2C19*2 and adverse events.

Adverse events	CYP2C19*2		*p*-value
	Wild (n = 43)	Heterozygous (n = 7)	
Re-intervention	3 (7.0%)	0 (0.0%)	1.000
Major amputation	0 (0.0%)	4 (57.1%)	< 0.001*
Non-fatal MI	1 (2.3%)	1 (14.3%)	0.263
Minor stroke	1 (2.3%)	2 (28.6%)	0.048*
Death	3 (7.0%)	0 (0.0%)	1.000

MI: myocardial infarction.

Discussion

Globally, the use of clopidogrel is recommended in combination with aspirin as a dual antiplatelet therapy as a mean to prevent vascular diseases. Despite the importance of clopidogrel, studies have demonstrated that about 30% of patients treated with clopidogrel do not effectively respond[18]. This phenomenon is known as "clopidogrel resistance," and its exact underlying mechanism is not known yet. Several hypotheses have been proposed over the mechanisms of clopidogrel resistance, and these hypotheses implicate both genetic (polymorphisms in the genes involved in the processing of clopidogrel in the body, such as *CYP2C19*) and nongenetic factors, such as gender, BMI, age, concomitant treatment, smoking status, and diseases[6].

A genome-wide association study has found that the polymorphisms of the CYP2C19 enzyme (the latter being the key predictor of altered clopidogrel response) account for only 12% of the variability observed in thienopyridine clopidogrel response[19]. Although other genetic and environmental factors may play an important role, Studies have indicated that certain nongenetic factors may affect the inhibitory effect of clopidogrel therapy on platelet function[20].

The present study aims to determine the frequency of the genetic polymorphism of *CYP2C19*2* and the contribution of this polymorphism and other nongenetic factors to clopidogrel response in an Egyptian population with PAD. Using LTA, a 22% incidence of clopidogrel resistance was observed among patients treated with clopidogrel following a vascular intervention, a finding that is consistent with rates already published in the literature (4%–44%)[21].

In the present study, the results showed that several clinical factors were associated with clopidogrel resistance. Univariate analysis demonstrated a statistically significant correlation between clopidogrel resistance and diabetes. Previous clinical trials have shown that patients with diabetes on clopidogrel treatment had worse clinical outcomes and increased thrombosis[22]. The latter might be due to insulin resistance in patients with uncontrolled diabetes. It is well established that type 1 (T1D) and 2 (T2D) diabetes adversely affect platelets via two pathways: an insulin receptor substrate (IRS)-1-dependent and IRS-1-independent pathway. The IRS-1-dependent pathway produces an increase in intracellular calcium concentrations, whereas the IRS-1-independent pathway decreases cellular sensitivity to nitric oxide and prostacyclin[23]. In addition, increased exposure to ADP and platelet turnover are known to lead to HTPR[24]. Moreover, it is noteworthy to mention that an increased expression of the glycoprotein IIb/IIIa on the platelet surface is reported to lead to platelet aggregation and decreased clopidogrel responsiveness[25].

Our results showed that high FBG and HbA1c levels were significantly correlated to clopidogrel resistance. Schuette et al[26] reported that ADP-PGE induced an aggregation of platelets positively correlated with FBG and HbA1c levels. The same authors have also documented a correlation between thrombogenicity and blood glucose levels in T2D. Furthermore, they have reported that a reduction in HbA1c levels was associated with a reduction in blood thrombogenicity. Our results contrasted with Al-Azzam et al[12].

In this study, no significant correlation between clopidogrel resistance and age, hypertension, or smoking was observed. In agreement with previous studies, Al-Azzam et al[12] have reported that age, hypertension, and smoking had no significant contribution to clopidogrel resistance. The blood pressure of our patients was tightly controlled. Therefore, we did not observe any correlation between hypertension and clopidogrel resistance. On the other hand, Yaseen et al[25] have found a significant association between hypertension and clopidogrel resistance, whereas Khalil et al[27] have suggested that age is significantly associated with nonresponse to clopidogrel.

Our results do not demonstrate a significant association of co-medication with clopidogrel resistance. The probable explanation could be the exclusion of other medication that might have provoked a diminished antiplatelet response due to a shared metabolic pathway via the CYP2C19, CYP3A4, or CYP2C9 isoenzymes.

As far as the patients' gender is concerned, female sex was previously suggested to influence the antiplatelet effects of clopidogrel[12,28]. We, on the other hand, did not observe any correlation between clopidogrel resistance and female sex in our study population. A meta-analysis of 79,613 patients has not managed to find any significant difference between males and females in clopidogrel resistance, a fact that supports our results[29].

There are few studies related to the incidence of adverse events in patients with PAD. In our study, no significant correlation was found between the two groups and the reintervention

rate. The limb salvage rate was significantly higher in the clopidogrel-sensitive group than in the clopidogrel-resistant one (97.4% *vs*. 72.7%, respectively; $p = 0.029$). Pastromas et al[30] have reported that the post PTA evaluation of the HPR by ADP can provide prognostic information on the incidence of target limb reintervention (TLR) and limb salvage rate in patients with PAD treated with clopidogrel and aspirin for 6 months. They have shown that the TLR rate was significantly greater in the clopidogrel-resistant group than in the clopidogrel-sensitive one (71.2% *vs*. 31.8%, respectively; $p < 0.001$). The limb salvage rate in their study was similar between the two groups: 98.3% in the clopidogrel-sensitive and 96.7% in the clopidogrel-resistant group ($p = 0.56$). Patients with PAD with a CYP2C19 loss-of-function allele and HPR have been reported to exert a reduced response to clopidogrel therapy with subsequent significantly higher risk of ischemic events[31]. The present study shows that CYP2C19 polymorphism has a strong association with clinical outcomes (major amputation and minor stroke). In addition, patients with clopidogrel resistance experienced a higher incidence of major amputation and death than clopidogrel responders. Therefore, the CYP2C19 genotype is an important predictor of adverse cardiovascular outcomes for patients with PAD treated with clopidogrel.

Multivariate analysis showed that the *CYP2C19*2* allele and a high BMI were the most powerful predictors of clopidogrel resistance. In agreement with previous studies, Karaźniewicz-Łada et al[32] have reported that the multivariate analysis suggests a significant effect of the *CYP2C19*2* allele ($p = 0.029$) and a BMI that is higher than 25 kg/m^2 ($p = 0.034$) on ADP-induced platelet aggregation. In fact, elevated BMI has been reported to be an independent predictor of suboptimal platelet response to clopidogrel[29]. Previous data also suggest that obese individuals bear an increased platelet function and altered clopidogrel metabolism, an observation that highlights the need to adjust clopidogrel dose in these patients. Finally, CYP3A4 activity has been shown to be reduced in overweight individuals[33].

As far as the genetic factors that predispose to clopidogrel resistance are concerned, it has been recently indicated that *CYP2C19* polymorphisms are a possible mechanism for clopidogrel resistance[34]. CYP2C19 plays a vital role in the transformation of clopidogrel into its active form, and *CYP2C19* polymorphisms are linked to clopidogrel response and ultimately cause thrombotic events. In our study, we identified a powerful contribution to clopidogrel resistance from the *CYP2C19*2* SNP, as **2* allele (*A* allele) carriers were found to be more resistant than **1* allele (*G* allele) carriers[35,36]. The allele **2* frequency in this study was found to be 7%, whereas other studies indicated that the allele **2* frequency has been reported to range from 25% to 30% in Caucasians[37], around 30% in Africans, and from 40% to 50% in East Asians. Hulot et al[38] have previously observed that 10 mM ADP-induced platelet aggregation was slowly reduced from baseline during clopidogrel treatment with a maintenance dose of 75 mg once daily in wild-type (**1/*1*) carriers but did not demonstrate any changes in heterozygous patients (**1/*2*).

We believe that the genotyping of the *CYP2C19*2* SNP might greatly enhance the clinical outcome of clopidogrel therapy. Furthermore, the undertaking of a platelet aggregation assay is recommended before and after clopidogrel treatment with a maintenance dose of 75 mg, as platelet reactivity is not a stable phenomenon and may require repetitive testing to confirm the clopidogrel resistance status of patients. Patients with clopidogrel resistance may benefit from alternative antiplatelet drugs. The use of novel and more potent drugs or a high clopidogrel maintenance dosing may be beneficial treatment options for antiplatelet therapy in *CYP2C19*2* carriers. Among the novel antiplatelet agents with the ability to resolve clopidogrel resistance are prasugrel and ticagrelor, both of which have recently been approved for clinical use. Prasugrel is a third-generation thienopyridine requiring only one step of hepatic metabolism; as a result, its active metabolites are produced faster and more efficiently, thus translating into a better pharmacodynamic effect[39]. Ticagrelor, on the other hand is a direct and reversible P2Y12 ADP receptor blocker that does not require hepatic metabolism and can achieve a greater range of platelet aggregation inhibition compared to clopidogrel[40].

Our study had some limitations. Firstly, this is a pilot study with limited patient numbers. As mentioned above, the objective of this novel study was to provide preliminary, exploratory observations of clopidogrel resistance in Egyptian patients with PVD that would serve a basis for the generation of hypotheses for future studies. Secondly, there might be several unmeasured demographic and/or clinical differences that we have failed to take into account.

Conclusions

Our findings indicate that several factors, including the carrying of a *CYP2C19*2* allele, obesity, and diabetes (especially uncontrolled diabetes), might contribute to the development of clopidogrel resistance. As a result, the risk of clinical adverse events may be increased during clopidogrel therapy. Our findings are suggestive of the need for the adoption of a more systematic platelet monitoring as a prerequisite in our attempt to reduce the incidence of clopidogrel resistance among high-risk patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This research did not receive any specific grant from funding agencies.

Acknowledgements

The authors would like to thank the health care professionals at AMUH for their participation and assistance in collecting patient data.

Ethics Approval

Approval was obtained from the Research Ethics Committee of the Faculty of Pharmacy of Damanhour University and the Ethics Committee of the AMUH in Egypt (Ref. No. 1218 PP 8).

References

1) Willey J, Mentias A, Vaughan-Sarrazin M, McCoy K, Rosenthal G, Girotra S. Epidemiology of lower extremity peripheral artery disease in veterans. J Vasc Surg 2018; 68: 527-535.e5.
2) Grifoni E, Gori AM, Giusti B, Valenti R, Migliorini A, Basili S, Paniccia R, Elmahdy MF, Pulli R, Pratesi C, Antoniucci D, Violi F, Marcucci R. On-treatment platelet reactivity is a predictor of adverse events in peripheral artery disease patients undergoing percutaneous angioplasty. Eur J Vasc Endovasc Surg 2018; 56: 545-552.
3) Schmit K, Dolor RJ, Jones WS, Vemulapalli S, Hasselblad V, Subherwal S, Heidenfelder B, Patel MR. Comparative effectiveness review of antiplatelet agents in peripheral artery disease. J Am Heart Assoc 2014; 3: e001330.
4) Lin J, Han Z, Wang C, Yi X, Chai Z, Zhou Q, Huang R. Dual therapy with clopidogrel and aspirin prevents early neurological deterioration in ischemic stroke patients carrying CYP2C19*2 reduced-function alleles. Eur J Clin Pharmacol 2018; 74: 1131-1140.
5) Idrissi HH, Hmimech W, Khorb NE, Akoudad H, Habbal R, Nadifi S. A synergic effect between CYP2C19*2, CYP2C19*3 loss-of-function and CYP2C19*17 gain-of- function alleles is associated with clopidogrel resistance among Moroccan acute coronary syndromes patients. BMC Res Notes 2018; 18: 11-46.
6) Notarangelo MF, Bontardelli F, Merlini PA. Genetic and nongenetic factors influencing the response to clopidogrel. J Cardiovasc Med (Hagerstown) 2013; 14; Suppl 1: S1-S7.
7) Janssen PW, ten Berg JM. Platelet function testing and tailored antiplatelet therapy. J Cardiovasc Transl Res 2013; 6: 316-328.
8) Gross L, Aradi D, Sibbing D. Platelet function testing in patients on antiplatelet medications. Semin Thromb Hemost 2016; 42: 306-320.
9) Guirgis M, Thompson P, Jansen S. Review of aspirin and clopidogrel resistance in peripheral arterial disease. J Vasc Surg 2017; 66: 1576-1586.
10) Botton MR, Lu X, Zhao G, Repnikova E, Seki Y, Gaedigk A, Schadt EE, Edelmann L, Scott SA. Structural variation at the CYP2C locus: characterization of deletion and duplication alleles. Hum Mutat 2019; 40: e37-e51.
11) Lee SJ. Clinical application of CYP2C19 pharmacogenetics toward more personalized medicine. Front Genet 2012; 3: 318.
12) Al-Azzam SI, Alzoubi KH, Khabour OF, Nusair MB, Al-Hadidi H, Awidi A, Saleh A. Factors that contribute to clopidogrel resistance in cardiovascular disease patients: environmental and genetic approach. Int J Clin Pharmacol Ther 2013; 51: 179-186.
13) Scott SA, Sangkuhl K, Stein CM, Hulot JS, Mega JL, Roden DM, Klein TE, Sabatine MS, Johnson JA, Shuldiner AR. Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clin Pharmacol Ther 2013; 94: 317-323.
14) Mega JL, Simon T, Collet JP, Anderson JL, Antman EM, Bliden K, Cannon CP, Danchin N, Giusti B, Gurbel P, Horne BD, Hulot JS, Kastrati A, Montalescot G, Neumann FJ, Shen L, Sibbing D, Steg PG, Trenk D, Wiviott SD, Sabatine MS. Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. JAMA 2010; 304: 1821-1830.
15) Núñez-Gil IJ, Bernardo E, Feltes G, Escaned J, Mejía-Rentería HD, De Agustín JA, Vivas D, Nombela-Franco L, Jiménez-Quevedo P, Macaya C, Fernández-Ortiz A. Platelet function in takotsubo cardiomyopathy. J Thromb Thrombolysis 2015; 39: 452-458.

16) Yang J, Yu Q, Xu Z, Zheng N, Zhong J, Li J, Liu Y, Xu H, Su J, Ji L, Chen X. Clopidogrel resistance is associated with DNA methylation of genes from whole blood of humans. Front Genet 2020; 11: 583215.

17) Hashemizadeh Z, Malek-Hosseini SA, Badiee P. Prevalence of CYP2C19 genetic polymorphism among normal people and patients with hepatic diseases. Int J Organ Transplant Med 2018; 9: 27-33.

18) Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RBl, Damcott CM, Pakyz R, Tantry US, Gibson Q, Pollin TI, Post W, Parsa A, Mitchell BD, Faraday N, Herzog W, Gurbel PA. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 2009; 302: 849-857.

19) Shen ZJ, Chen XA, Wang YJ, Guo W, Chen TJ, Wang XL. [Correlation between CYP2C19 gene polymorphism with clopidogrel resistance and distribution of Chinese medicine syndrome in 229 acute coronary syndrome patients]. Zhongguo Zhong Xi Yi Jie He Za Zhi 2017; 37: 291-296.

20) Ali Z, Elewa H. The effect of CYP2C19 and non-genetic factors on clopidogrel responsiveness in the MENA Region: A systematic review. Clin Appl Thromb Hemost 2019; 25: 1076029619875520.

21) Dupont AG, Gabriel DA, Cohen MG. Antiplatelet therapies and the role of antiplatelet resistance in acute coronary syndrome. Thromb Res 2009; 124: 6-13.

22) Angiolillo DJ, Capranzano P, Desai B, Shoemaker SB, Charlton R, Zenni MM, Guzman LA, Bass TA. Impact of P2Y12 inhibitory effects induced by clopidogrel on platelet procoagulant activity in type 2 diabetes mellitus patients. Thromb Res 2009; 124: 318-322.

23) Kumbhani DJ, Marso SP, Alvarez CA, McGuire DK. State-of-the-Art: hypo-responsiveness to oral antiplatelet therapy in patients with type 2 diabetes mellitus. Curr Cardiovasc Risk Rep 2015; 9: 4.

24) Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279: 3254-3264.

25) Yaseen IF, Farhan HA, Abbas HM. Clopidogrel non-responsiveness in patients undergoing percutaneous coronary intervention using the VerifyNow test: frequency and predictors. Eur J Hosp Pharm 2019; 26: 113-116.

26) Schuette C, Steffens D, Witkowski M, Stellbaum C, Bobbert P, Schultheiss HP, Rauch U. The effect of clopidogrel on platelet activity in patients with and without type-2 diabetes mellitus: a comparative study. Cardiovasc Diabetol 2015; 14: 15.

27) Khalil BM, Shahin MH, Solayman MH, Langaee T, Schaalan MF, Gong Y, Hammad LN, Al-Mesallamy HO, Hamdy NM, El-Hammady WA, Johnson JA. Genetic and nongenetic factors affecting clopidogrel response in the Egyptian population. Clin Transl Sci 2016; 9: 23-28.

28) Sharma RK, Erickson SW, Sharma R, Voelker DJ, Reddy HK, Dod H, Marsh JD. Platelet function testing to predict hyporesponsiveness to clopidogrel in patients with chest pain seen in the emergency department. Vasc Health Risk Manag 2013; 9: 187-193.

29) Angiolillo DJ, Fernández-Ortiz A, Bernardo E, Barrera Ramírez C, Sabaté M, Fernandez C, Hernández-Antolín R, Escaned J, Alfonso F, Macaya C. Platelet aggregation according to body mass index in patients undergoing coronary stenting: should clopidogrel loading-dose be weight adjusted? J Invasive Cardiol 2004; 16: 169-174.

30) Pastromas G, Spiliopoulos S, Katsanos K, Diamantopoulos A, Kitrou P, Karnabatidis D, Siablis D. Clopidogrel responsiveness in patients undergoing peripheral angioplasty. Cardiovasc Intervent Radiol 2013; 36: 1493-1499.

31) Guo B, Tan Q, Guo D, Shi Z, Zhang C, Guo W. Patients carrying CYP2C19 loss of function alleles have a reduced response to clopidogrel therapy and a greater risk of in-stent restenosis after endovascular treatment of lower extremity peripheral arterial disease. J Vasc Surg 2014; 60: 993-1001.

32) Karaźnewicz-Łada M, Danielak D, Rubiś B, Burchardt P, Oszkinis G, Główka F. The influence of genetic polymorphism of Cyp2c19 isoenzyme on the pharmacokinetics of clopidogrel and its metabolites in patients with cardiovascular diseases. J Clin Pharmacol 2014; 54: 874-880.

33) Wang L, Wang X, Chen F. Clopidogrel resistance is associated with long-term thrombotic events in patients implanted with drug-eluting stents. Drugs R D 2010; 10: 219-224.

34) Tekkeşin Aİ, Kaya A, Çakıllı Y, Türkkan C, Hayıroğlu Mİ, Borklu EB, Kalenderoğlu K, Gümüşdağ A, Yıldırımtürk Ö, Bozbeyoğlu E, Tatlısu MA, Alper AT. The first six-month clinical outcomes and risk factors associated with high on-treatment platelet reactivity of clopidogrel in patients undergoing coronary interventions. Anatol J Cardiol 2016; 16: 967-973.

35) Cedillo-Salazar FR, Martínez-Jacobo L, Pérez-Páramo YX, Cerda-Flores R, Martínez LE, Jaime-Pérez JC, Moreno-Treviño MG, Pérez-Rodríguez E, Bosques-Padilla FJ, Cedillo-Avila M, Cedillo-Avila MA, Zamudio-Osuna M. Association of CYP2C19*2 polymorphism with clopidogrel resistance among patients with high cardiovascular risk in Northeastern Mexico. Arch Cardiol Mex 2019; 89: 324-329.

36) Su Q, Li J, Tang Z, Yang S, Xing G, Liu T, Peng H. Association of CYP2C19 Polymorphism with clopidogrel resistance in patients with acute coronary syndrome in China. Med Sci Monit 2019; 25: 7138-7148.

37) Miao J, Liu R, Li Z. Cytochrome P-450 polymorphisms and response to clopidogrel. N Engl J Med 2009; 360: 2250-2251.

38) Hulot JS, Bura A, Villard E, Azizi M, Remones V, Goyenvalle C, Aiach M, Lechat P, Gaussem

P. Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood 2006; 108: 2244-2247.

39) Gajos G, Zalewski J, Nessler J, Żmudka K, Undas A, Piwowarska W. Polyunsaturated omega-3 fatty acids improve responsiveness to clopidogrel after percutaneous coronary intervention in patients with cytochrome P450 2C19 loss-of-function polymorphism. Kardiologia Polska (polish heart journal) 2012; 70: 439-445.

40) Amsterdam EA, Wenger N, Brindis R, Casey Jr D, Ganiats T, Holmes Jr D. 2014 AHA/ACC Guideline for the management of Patients With Non–ST-Elevation Acute coronary syndromes. a report of the American College of Cardiology/ American Heart Association Task Force on Practice Guidelines 2014; 2014: 64.