Abstract. – OBJECTIVE: The current study was conducted to determine the distribution of genetic polymorphisms in CYP2C19 in Iraqi patients and their role in inter-individual variability of clopidogrel efficacy.

PATIENTS AND METHODS: A prospective controlled study was done on 100 patients under high risk of cardiovascular diseases who started clopidogrel prophylactic therapy. Polymerase chain reaction-restriction fragment length polymorphism method was used to determine the existence of the CYP2C19 gene mutation. Vasodilator-stimulated phosphoprotein (VASP) index baseline besides one-month post-therapy was analyzed by dual-color flow cytometry analysis.

RESULTS: Eight gene mutations of CYP2C19 were found (*1/*1), (*1/*2), (*1/*3), (*1/*8), (*1/*17), (*2/*2), (*2/*4), and (*3/*3) with higher prevalent CYP2C19*1 allele. Homozygous CYP2C19*1 allele was shown to be the rapid metabolizer comparing to the heterozygous CYP2C19*1 allele, whereas, CYP2C19*2 and CYP2C19*3 were resistant alleles and were present in 28% of patients. The analysis of VASP phosphorylation produces accurate inter-individual response variability in platelets inhibition by antiplatelet drugs.

CONCLUSIONS: In vitro gene analysis and VASP index improve the clinical outcome of a patient candidate to clopidogrel as prophylaxis in cardiovascular events.

Key Words: Genetic polymorphisms, CYP2C19, Clopidogrel, VASP index, Cardiovascular diseases.

Introduction

Antiplatelet therapy has a central role in the prevention of coronary artery diseases. Clopidogrel is one of many antiplatelet drugs frequently considered for unrestrained use in patients with peripheral artery diseases. It can decrease mortality and improve cardiovascular outcomes without increases bleeding tendency in patients with acute coronary syndrome. Cannon et al. indicated that clopidogrel in a dosage of 75 mg daily has a significant reduction in cardiovascular endpoints by approximately 25%.

Current approaches in patients undergoing optional percutaneous coronary intervention (PCI) include administration of aspirin, a P2Y12 antagonist (clopidogrel, prasugrel, or ticagrelor), an antithrombin agent, and occasionally a glycoprotein (GP) IIb/IIIa receptor antagonist. Clopidogrel inhibits adenosine diphosphate (ADP) binding to the P2Y12 platelet receptor; thus, prevents platelet activation and, consequently, the process of aggregation.

There are many well-known factors associated with pharmacokinetics and pharmacodynamics variability of clopidogrel, including patient compliance, diet, smoking, alcohol, demographic factors, drug-drug interaction (enzyme inducer or enzyme inhibitor), and platelet hyperactivity. Moreover, the genetic polymorphisms of
CYP2C19 polymorphisms and clopidogrel

Patients and Methods

Patients
A prospective controlled study included 100 patients admitted to the Baghdad Teaching Hospital, Medical City, Baghdad, Iraq from June 2019 to January 2020, aged between 40 to 65 years old, not on clopidogrel, antiplatelet or anticoagulant therapy. Those patients are candidates to use clopidogrel as prophylaxis against cardiovascular diseases. All patients are informed of the research steps and objectives, and they are given written informed consent to sign before inclusion.

The patients excluded from the present study are those contraindicated for antiplatelet therapy, smokers, pregnant women, patients concomitantly using drugs causing enzyme inducers or inhibitors, patients with a history of pathological bleeding, and patients who have VASP index below 55%. The patient’s demography was documented, including age, sex, blood pressure, diabetes, heart failure, and myocardial infarction (Table I).

Research Method
Before the initiation of clopidogrel therapy, all patients had their blood samples from the antecubital vein by non-traumatic venipuncture, and their VASP index baseline was analyzed. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the existence of the CYP2C19 gene mutation. All patients started with clopidogrel 75 mg daily and reevaluation was established after 30 days.

Efficacy of Clopidogrel by VASP Index
Clopidogrel efficacy was evaluated for one-month and the efficacy was tested within 48 h after collection of blood. Blood samples were stored...
at room temperature (18-25°C) in trisodium citrate (0.129 M) in the ratio of 9:1 (blood to citrate) and were analyzed using PLT VASP/P2Y12 kit (Biocytex, Marseille, France) by following company guidelines. Initially, blood samples were incubated with prostaglandin E1 (PGE1) alone or PGE1 with ADP. All samples were labeled with clone 16C2 by indirect no-wash immunofluorescence. Dual-color flow cytometry analysis (Coulter EPICS XL cytometer; Beckman Coulter Inc., Fullerton, CA, USA) was used to compare the results of two samples to inhibit VASP phosphorylation by ADP. The platelet reactivity index was measured by the ratio, $100 \times \frac{[\text{Mean fluorescence intensity PGE1} - \text{Mean fluorescence intensity ADP+PGE1}]}{\text{Mean fluorescence intensity PGE1}}$. The cut-off value of the VASP index for detecting clopidogrel activity was 55%\(^\text{12}\).

**Polymorphism in CYP2C19 Gene**

SNPs of interest were detected using primer extension technique, as described by Adithan et al\(^\text{13}\). Patients were grouped according to the CYP2C19 genotypes to evaluate their effect on the VASP index.

**Ethics Statement**

The Research Ethics Committee at Baghdad Teaching Hospital, Baghdad, Iraq approved this study protocol (Ethical approval No: 1448 on May-2019). Further, an informed verbal consent was obtained from all the study participants to include in the study.

**Statistical Analysis**

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Statistically significant values are considered at $p < 0.05$. The mean-standard deviation is used to express variables. Frequency and percentage are used to represent the categorical variables. A Chi-square test was used to assess independent variability. The normal platelet reactivity without clopidogrel is above 60-70%\(^\text{14}\).

**Results**

**Genotype Distribution**

The genotype results are shown in Figure 2. For total 100 patients, eight gene mutations of
CYP2C19 polymorphisms and clopidogrel

CYP2C19 were found (*1/*1), (*1/*2), (*1/*3), (*1/*8), (*1/*17), (*2/*2), (*2/*4), and (*3/*3). The distribution of these mutations was: 72 patients found to have CYP2C19 *1 gene and 28 patients were detected to have CYP2C19 *2 and CYP2C19 *3 gene mutation.

VASP phosphorylation

A total of 100 patients had a baseline VASP index of 69.13 ± 5.47% before starting clopidogrel therapy. Thirty days after clopidogrel therapy in a dose of 75 mg/day resulted in a significant reduction in the VASP index to 49.58 ± 11.68 (% = 0.001). The result indicated a small variation in the VASP index between the patients before therapy, ranging from 50.37 to 80.42; conversely, higher variation seen after therapy with a range of 35.11 to 78.17 (Table II).

The distribution of the CYP2C19 gene mutation related to the VASP index was illustrated in Table III and Figure 3. One month after clopidogrel therapy, the VASP index significantly decreased in patients with CYP2C19*1 allele. Homozygous CYP2C19*1 allele was shown to be the rapid metabolizer comparing to the heterozygous CYP2C19*1 allele. In contrast, patients with homozygous CYP2C19*2 alleles show no significant effect on the VASP index (% = 0.84), while heterozygous CYP2C19*2 alleles significantly reduced VASP index. For patients with homozygous CYP2C19*3 alleles, the effect on the VASP index was non-significant.

Discussion

Genetic polymorphism of CYP2C19 is widely distributed and results in wide variability in clopidogrel therapeutic effect. The most common polymorphism of CYP2C19 is the genotype of CYP2C19*1, CYP2C19*2, CYP2C19*3, and rare type of CYP2C19*4, CYP2C19*5, CYP2C19*8. The activity of CYP2C19 was expressed as fast, intermediate, and slow metabolizer. Homozygous type of CYP2C19*1 allele (CYP2C19*1/*1) is a rapid metabolizer, whereas heterozygous types of CYP2C19*1/*2, CYP2C19*1/*3, CYP2C19*1/*4, and CYP2C19*1/*8 alleles exhibit slow metabolizing activity. The homozygous CYP2C19*2/*2 and CYP2C19*3/*3, as well as the heterozygous CYP2C19*2/*3 and CYP2C19*2/*4 alleles exhibit slow metabolizing activity. The high risk

Table II. VASP index before and after clopidogrel therapy.

<table>
<thead>
<tr>
<th>VASP index (total patients)</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>69.13 ± 5.47</td>
<td>50.37 - 80.42</td>
<td>30.05</td>
<td>.001</td>
</tr>
<tr>
<td>After treatment</td>
<td>49.58 ± 11.68</td>
<td>35.11 - 78.17</td>
<td>43.06</td>
<td></td>
</tr>
</tbody>
</table>
of major adverse cardiovascular events (MACE) associated with clopidogrel use in CYP2C19 loss of function allele carriers suggests that the use of genotype-guided dual antiplatelet therapy (DAPT) in practice may improve clinical outcomes. Friedman et al. consistently demonstrate that a genotype-guided DAPT reduces the risk of MACE in ≈30% of the US population, with less risk of bleeding tendency. Moreover, the use of genotype-guided antiplatelet therapy improves patient outcomes following PCI. Similar results were found in patients with cerebrovascular diseases using clopidogrel. The relationship between CYP2C19 metabolizer status and clinical outcomes was extensively studied. CYP2C19 rapid and ultrarapid metabolizers treated with clopidogrel show high plasma concentration of clopidogrel active metabolite and increased inhibition of platelet aggregation, as well as higher bleeding risk and lower MACE risk among clopidogrel-treated patients. Therefore, assessment of patients’ clinical risk is required to determine if a patient is a poor metabolizer before starting clopidogrel therapy. Genetic testing is essential for patients believed to be at moderate or high risk for poor outcomes, like patients undergoing elective high-risk PCI procedures.

In the current study, the CYP2C19 gene alleles were three types (CYP2C19*1, CYP2C19*2, and CYP2C19*3). The higher frequency was CYP2C19*1 (72%), whereas CYP2C19*2 and CYP2C19*3 were less frequent (22% and 6%, respectively). Different results have been reported in other studies (Table IV). The frequency of CYP2C19*1 alleles found in the present study was close to Iranian and Saudi Arabian populations but lower than the frequencies in the Jordanian, Egyptian, and Turkish people (84.0%, 87.8%, and 88.0%, respectively). CYP2C19*2 alleles frequencies in Iranian, Saudi Arabian, and Turkish people were 12.0%, 11.2%, and 11.6%, respectively, are closed to our finding, but less than Jordanian (16.0%) and more than Egyptian (10.9%) population. CYP2C19*3 alleles were absent in Saudi Arabian and Jordanian populations, as well as the Iranian and Turkish populations. These individualized differences could be due to variability in the studied populations and the small size of our study (one of its limitations).

Platelet function testing is a promising alternative option for patients with suboptimal efficacy of clopidogrel. The latest American Heart Association (AHA) guideline recommends that patients under high risk of thrombosis, such as bifurcating left main, unprotected left main, or last patent coronary vessel, higher dose of clopidogrel (150 mg daily) is considered if the inhibition of platelet aggregation was less than 50%.

### Table III. CYP2C19 gene mutation in relation to the VASP index.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VASP index</th>
<th>Difference of mean</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before clopidogrel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One month after clopidogrel treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C19 (*1/*1) (N=20)</td>
<td>66.08 ± 5.09</td>
<td>37.37 ± 2.52</td>
<td>28.71</td>
</tr>
<tr>
<td>CYP2C19 (*1/*2) (N=22)</td>
<td>70.35 ± 4.07</td>
<td>45.32 ± 0.34</td>
<td>25.03</td>
</tr>
<tr>
<td>CYP2C19 (*1/*3) (N=20)</td>
<td>71.36 ± 5.43</td>
<td>43.37 ± 1.17</td>
<td>27.99</td>
</tr>
<tr>
<td>CYP2C19 (*1/*8) (N=8)</td>
<td>68.52 ± 3.69</td>
<td>48.09 ± 1.36</td>
<td>20.43</td>
</tr>
<tr>
<td>CYP2C19 (*1/*17) (N=2)</td>
<td>75.42</td>
<td>49.53</td>
<td>25.89</td>
</tr>
<tr>
<td>Total (N=72)</td>
<td>69.41 ± 5.12</td>
<td>42.94 ± 4.13</td>
<td>26.47</td>
</tr>
<tr>
<td>CYP2C19 (*2/*2) (N=8)</td>
<td>67.15 ± 7.84</td>
<td>67.96 ± 2.77</td>
<td>0.81</td>
</tr>
<tr>
<td>CYP2C19 (*2/*4) (N=14)</td>
<td>66.21 ± 2.82</td>
<td>59.81 ± 3.70</td>
<td>6.4</td>
</tr>
<tr>
<td>Total= 22</td>
<td>66.81 ± 6.28</td>
<td>64.87 ± 4.98</td>
<td>1.94</td>
</tr>
<tr>
<td>CYP2C19 (*3/*3)</td>
<td>74.28 ± 2.21</td>
<td>73.15 ± 2.12</td>
<td>1.13</td>
</tr>
</tbody>
</table>

VASP is a widely distributed vertebrate intracellular protein activated by phosphorylation that controls all vasodilator-stimulated phosphor-pro
CYP2C19 polymorphisms and clopidogrel

Platelet activation begins after adhesion to the damaged vascular wall, mainly regulated by endothelial factors, nitric oxide (NO), and prostacyclin. These factors activate soluble guanylyl cyclase that subsequently increases platelet cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP). The NO/cGMP and prostacyclin/cAMP signaling are mainly targeting the cGMP-dependent protein kinase I and cAMP-dependent protein kinase. The common substrate of these kinases is the VASP, which is distributed in a variety of cells and tissues in addition to the platelets. In platelets, VASP is strategically located at the intersection of two major inhibitory pathways.

Studies showed the direct correlation of VASP phosphorylation with the inhibition of platelet activation and the expression of GP Iib/IIa receptors. Moreover, in VASP-deficient mice, platelets show more expression of P-selectin and GP Iib/IIa receptors. Meanwhile, the antagonism of platelet ADP receptor P2Y12 by clopidogrel was found to suppress VASP phosphorylation and prevent platelet aggregation. Therefore, VASP is associated with the regulation of platelet stimulation both in vitro and in vivo. In accordance with these observations, the inhibition of platelet aggregation by antiplatelet drugs can impair VASP knockout. Consequently, VASP phosphorylation could be used as a marker for antiplatelet activities.

One of the most common principles to describe inter-individual variability in patients treated with P2Y12 receptor antagonists is via the VASP index as explained by Bal Dit Sollier et al. As clopidogrel inhibits platelet aggregation by blocking ADP-dependent platelets receptor activation, the present study’s result allows for two main conclusions: firstly, the measurement of VASP phosphorylation by cytometry was useful for determination of reduction in platelets’ activity by clopidogrel therapy. Secondly, the analysis of VASP phosphorylation produces accurate inter-individual response variability in platelets’ inhibition by antiplatelet drugs.

Our finding illustrates three types of genotype distributed in the studied patients. The most prevalent was the CYP2C19*1 allele that demonstrates an excellent response to clopidogrel therapy with a significant reduction in the VASP phosphorylation index (<55%). This result supported by Samoš et al found a good activity of P2Y12 receptor antagonist measured as the VASP phosphorylation index. Meanwhile, the clopidogrel loading dose adjustment significantly improves the clinical outcome after PCI in patients using the VASP index. The homozygous alleles of the CYP2C19*1 allele found to be rapid metabolizers comparing to the heterozygous alleles. This was illustrated by a more significant reduction in the VASP index for homozygous vs. heterozygous alleles. Our result was consistent with many global studies.

Two groups of patients were found to be resistant to clopidogrel therapy carrying CYP2C19*2 and CYP2C19*3 alleles that have a VASP phosphorylation index >55%. This result supported by Nasyuhana et al concluded that the CYP2C19*2 allele was related to a decrease in platelet responsiveness to clopidogrel. Despite the higher prevalence of CYP2C19*1 allele, the occurrence of total resistant alleles was 28% of the separately studied patients; CYP2C19*2 and CYP2C19*3 were founded in 22% and 6% of patients, respectively. For patients with CYP2C19*2 alleles, both homozygous and heterozygous alleles seemed re-

### Table IV. Alleles and genotype frequencies of CYP2C19 in different countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>No.</th>
<th>Reference</th>
<th>CYP2C19 allele frequency (%)</th>
<th>CYP2C19 genotype frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iraq</td>
<td>100</td>
<td>Current study</td>
<td>66.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Iran</td>
<td>84</td>
<td>24</td>
<td>71</td>
<td>12</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>201</td>
<td>25</td>
<td>62.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Jordan</td>
<td>78</td>
<td>26</td>
<td>84.0</td>
<td>16</td>
</tr>
<tr>
<td>Egypt</td>
<td>120</td>
<td>27</td>
<td>87.8</td>
<td>10.9</td>
</tr>
<tr>
<td>Turkish</td>
<td>404</td>
<td>28</td>
<td>88</td>
<td>11.6</td>
</tr>
</tbody>
</table>

*Note: The data represents the frequency (%) of each CYP2C19 allele and genotype in different countries.*
sistant to clopidogrel therapy. Mega et al argued that the effect of different clopidogrel dosing and found that a subset of CYP2C19*2 homozygous patients, even with a dose of 300 mg of clopidogrel was unable to accomplish on-treatment platelet reactivity. Conversely, the high clopidogrel-loading dose of 900 mg was able to adequately lower on-treatment platelet reactivity.

Other potential mechanisms of genetic variability of clopidogrel activity, which should be considered, are polymorphisms in the ABCB1 genes encoding P-glycoprotein (an efflux transporter) and paraoxonase-1 (PON1) (the rate-limiting enzyme in clopidogrel metabolic activation). Momary et al. studied the effect of polymorphism ABCB1 and R isoforms found to cause different rates of clopidogrel activation. Furthermore, Polasek et al. found lower plasma concentrations of the active metabolite of clopidogrel and lower platelet inhibition in association with a lower PON1 plasma activity.

The polymorphism in the P2Y12 receptor for clopidogrel and its effector, glycoprotein IIb/IIIa, could be another cause for variability in clopidogrel effect. A study conducted on 375 patients with ischemic stroke did not show any association between polymorphisms of P2Y12 or GPIIb/IIIa and clinical outcomes. Besides, Lev et al. and Floyd et al. did not find significant associations between polymorphisms in P2Y12, P2Y1, and GPIIb/IIIa with responsiveness clopidogrel in patients with cardiovascular diseases. In contrast, recent studies have shown that SNPs in P2Y12 and GPIIa genes may affect antiplatelet drug responsiveness. These mechanisms need further investigation to confirm their role in clopidogrel efficacy.

Conclusions

An in vitro genotype analysis can predict the antiplatelet activity of clopidogrel and improves clinical outcomes in the treatment setting with PCI or patients with cardiovascular diseases. The CYP2C19*1 has rapid metabolic activity and results in the good antiplatelet activity of clopidogrel, whereas the CYP2C19*2 and CYP2C19*3 are characterized by poor platelet responsiveness to clopidogrel and may, therefore, be an essential genetic indicator to therapeutic failure of clopidogrel in the clinical settings. Long-term trials with large-scale data are necessary to determine the prevalence of other CYP2C19 alleles and their precise role in clopidogrel efficacy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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