Cytochrome P450 2C19 loss-of-function polymorphism, but not CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with response variability to dual antiplatelet treatment in high-risk vascular patients

Betti Giustia, Anna Maria Gori, Rossella Marcuccia, Claudia Saracini, Ilaria Sestinia, Rita Paniccia, Serafina Valente, Davide Antoniucci, Rosanna Abbate and Gian Franco Gensini

Objectives The aim of this study was to evaluate the effect of polymorphisms affecting the clopidogrel metabolism (CYP3A4 IVS10 + 12G/A and CYP2C19*2) and the P2Y12 receptor (P2Y12 T744C) on modulating platelet function in acute coronary syndrome patients on dual antiplatelet treatment.

Background Residual platelet reactivity (RPR) phenomenon on antiplatelet therapy requires clarification. P2Y12 T744C, CYP3A4 IVS10 + 12G/A and, in healthy individuals only, CYP2C19*2 polymorphisms have been investigated; however, the influence on platelet reactivity in a large population of high-risk vascular patients on dual antiplatelet treatment has not yet been elucidated.

Methods A total of 1419 acute coronary syndrome patients on dual antiplatelet treatment were studied. Platelet function was evaluated by platelet-rich plasma aggregation. Electronic nanochips and restriction-fragment length polymorphism were used for analysis of polymorphisms.

Results Only CYP2C19*2, out of the three investigated polymorphisms, is associated with higher platelet reactivity. Carriers of the *2 allele had significantly higher platelet aggregation values after arachidonic acid (AA; \( P = 0.043 \)), 2 \( \mu \)mol/l adenosine 5’ diphosphate (ADP; \( P < 0.0001 \)) and 10 \( \mu \)mol/l ADP (\( P = 0.001 \)) stimuli. The genotype distribution of CYP2C19*2 polymorphism significantly differed between patients with and without RPR, as evaluated by 10-\( \mu \)mol/l ADP-induced platelet aggregation (\( P = 0.002 \)) and by AA-induced platelet aggregation (\( P = 0.045 \)). At the multivariate linear regression analysis, the CYP2C19*2 polymorphism remained a significant and independent risk factor for dual antiplatelet treatment variability.

Conclusions This study demonstrates, for the first time, that the *2 CYP2C19 allele is associated with higher platelet aggregability and RPR in high-risk vascular patients on dual antiplatelet treatment. These findings can have a significant impact on the future design of pharmacogenetic antiaggregant strategies for high-risk vascular patients on dual antiplatelet treatment. Pharmacogenetics and Genomics 17:1057–1064 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: acute coronary syndrome, ADP receptor P2Y12, cytochrome P450, dual antiplatelet treatment, pharmacogenetics, polymorphism, residual platelet reactivity

Introduction Platelet activation and aggregation play an important role in the pathogenesis of arterial thrombosis leading to acute coronary syndromes (ACS) and in thrombotic complications during and after percutaneous coronary interventions (PCI) [1,2].

Clopidogrel and acetylsalicylic acid (ASA) have become the gold standard therapy for preventing stent thrombosis in patients undergoing PCI, and for reducing major adverse cardiovascular events in patients with non-ST-segment elevation ACS [3,4]; however, there is a broad variability in individual responses to dual antiplatelet treatment [5–10]. Previous studies estimated that adequate antiplatelet effects are not achieved in 5–45% of patients taking ASA, and in 4–30% of patients taking clopidogrel [5,7–10]. Several studies investigated the clinical implications of antiplatelet drug effect resistance in chronic coronary artery disease (CAD) or stent thrombosis [6,9,11–15]. Recently, we demonstrated that post-PCI residual platelet reactivity (RPR) is an independent predictor of 1-year major adverse coronary events in patients with acute myocardial infarction (MI) [16] and of stent thrombosis in patients receiving drug-eluting stents [17].
Among the possible mechanisms of RPR, genetic polymorphisms could play a pivotal role in determining the individual susceptibility to antiplatelet drug response. Several polymorphisms in different genes coding platelet components [glycoprotein (Gp)Ia, GpIIa, GpIb-alpha, GpVI, platelet adenosine 5’ diphosphate (ADP) receptor P2Y12 (P2Y12), P-selectin, cyclooxygenase (COX)-1, COX-2] or cytochrome P450 enzyme isoforms [cytochrome P450-3A4, −3A5] have been proposed and investigated [18–20].

The active metabolite of clopidogrel, which irreversibly blocks platelet ADP P2Y12 receptors, arises from complex biochemical reactions [21–23] involving several cytochrome P450 (CYP) isoforms [18,22]. Variability in the catalytic activity of these isoforms, as well as in the structure of the P2Y12 receptor, might affect the pharmacodynamic action of clopidogrel.

In two recent studies [24,25], the CYP2C19*2 allelic variant (681A allele) encoding a deficient drug-metabolizing enzyme CYP2C19 [26] was associated with impaired platelet inhibition to clopidogrel in healthy men [24,25]. No data are available on the possible role of this genetic variant in affecting platelet response to therapy in ACS patients.

Moreover, in 82 patients with stable CAD using combined clopidogrel and aspirin therapy, the IVS10 + 12G/A polymorphism of the CYP3A4 gene was shown to modulate the expression of activated GP IIb/IIIa after 2 μmol/l ADP, but not the platelet aggregation profile [27]. As concerns the P2Y12 receptor gene, two haplotypes each composed of four polymorphisms in total linkage disequilibrium were identified: the minor haplotype was found to be associated with increased in-vitro ADP-induced platelet aggregation in healthy individuals [28]. This datum was not confirmed in patients on either the dual antiplatelet therapy (clopidogrel plus aspirin) or on aspirin alone [29,30].

The aim of this study was to evaluate the effect of the polymorphisms affecting the clopidogrel metabolism (CYP3A4 IVS10 + 12G/A and CYP2C19*2) and the P2Y12 receptor (P2Y12 T744C) on modulating platelet function in ACS patients on dual antiplatelet treatment.

Materials and methods

Study population

The study population included 1419 consecutive patients admitted to the Coronary Care Units of the Azienda Ospedaliero-Universitaria Careggi, University of Florence with diagnosis of ACS. Acute MI was diagnosed on the basis of an increase in creatine kinase MB isoenzyme to at least twice the upper normal limits (3.6 ng/ml), and/or elevated cardiac troponin I (cTnI) (> 0.15 ng/ml) levels with at least one of the following: acute onset of prolonged (≥ 20 min) typical ischemic chest pain; ST-segment elevation of at least 1 mm in two or more contiguous electrocardiographic leads; ST-depression of ≥ 0.5 mm, 0.08 s after the J point in ≥ 2 contiguous leads or T waves inversion > 1 mm in leads with predominant R waves. All patients underwent coronary angiography performed by the Judkins’ technique and primary PCI. Patients were considered to have hypertension if they had been diagnosed as hypertensive according to the European Society of Hypertension/European Society of Cardiology guidelines or were taking anti-hypertensive drugs. Dyslipidemia was defined according to the Third report of the National Cholesterol Education Program, and diabetes according to the American Diabetes Association.

The exclusion criteria included history of bleeding diathesis, platelet count ≤ 100 000/mm³, hematocrit ≤ 30% and serum creatinine ≥ 4.0 mg/dl.

All patients received a loading dose of 600 mg of clopidogrel orally before the procedure and of 500 mg of ASA intravenously, followed by 75 mg of clopidogrel and 100 mg of ASA daily. Unfractioned heparin 70 IU/kg was used during the procedure as an anticoagulant. The use of glycoprotein (Gp) IIb/IIIa inhibitors was at the discretion of the operating surgeon. For patients receiving both the loading dose of clopidogrel and the IIb/IIIa inhibitor in the catheterization laboratory, blood samples were obtained after 6 days.

Informed written consent was obtained from all patients and the study was approved by the local Ethical Review Board.

Blood sampling

Venous blood samples were taken from each patient 24 h after PCI intervention in tubes containing 3.2% trisodium citrate.

Assessment of platelet aggregation on platelet-rich plasma

Platelet aggregation was assessed using platelet-rich plasma (PRP) by the turbidimetric method in a four-channel aggregometer (APACT 4, Labor Biomedical Technologies GmbH, Ahrensburg, Germany). Platelet agonist included 2 μmol/l ADP, 10 μmol/l ADP and 0.5 mg/ml arachidonic acid (AA). PRP was obtained as a supernatant after centrifugation of citrated blood at 800 rpm for 10 min. Platelet-poor plasma (PPP) was obtained by a second centrifugation of the blood fraction at 2500 rpm for 10 min. Light transmission was adjusted to 0% with PRP and to 100% with PPP for each measurement. Curves were recorded for 6 min and analyzed according to international standards. According to the Born’s method, platelet aggregation was...
determined as the maximal percent change in light transmittance from baseline, using PPP as reference, in response to different stimuli (2 and 10 μmol/l ADP, and AA). Platelet count was assessed at all time points to ensure that the degree of platelet function was not biased by the number of platelets.

For antiplatelet response, the following definitions were used:

1. RPR evaluated by 10-μmol/l ADP-induced platelet aggregation (10-μmol/l ADP-RPR) = 10-μmol/l ADP-induced platelet aggregation ≥ 70% [17,31];
2. RPR evaluated by 0.5 mg/ml AA-induced platelet aggregation (AA-RPR) = AA-induced platelet aggregation > 20% [8].

**DNA extraction**

Genomic DNA was isolated from whole blood by using the FlexiGene DNA kit (Qiagen GmbH, Hilden, Germany).

**T744C P2Y12 gene polymorphism detection by electronic microchip**

P2Y12 gene sequences were obtained from GeneBank (www.ncbi.nlm.nih.gov, accession number NM_176876). The P2Y12 T744C polymorphism (rs2046934) has been evaluated by using the NanoChip Molecular Biology Workstation and the NanoChip cartridge (Nanogen, San Diego, California, USA). The protocol was extensively described in a previous paper [32]. The primers used for polymerase chain reaction (PCR), the oligonucleotide reporters and stabilizer for the hybridization reaction and the melting and stringency temperatures are reported in Table A (online supplemental data). The software of the system directly assigned the genotype to each sample.

**CYP2C19*2 (G681A CYP2C19) and IVS10 + 12G/A CYP3A4 gene polymorphisms detection by restriction fragment length polymorphism analysis**

CYP2C19 and CYP3A4 gene sequences were obtained from GeneBank (www.ncbi.nlm.nih.gov, accession number NM_000769 and NM_017460, respectively). The presence of the CYP2C19*2 (rs4244285) and CYP3A4 IVS10 + 12G/A (rs2244280) polymorphisms was determined by PCR–restriction fragment length polymorphism (RFLP).

The amplification of the sequences has been performed through PCR reaction in an MJ thermocycler (MJ Research, Waltham, Massachusetts, USA) with the following settings: one denaturation cycle at 94°C for 5 min, 39 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The reaction has been performed in a final volume of 25 μl with 100 ng of genomic DNA, 0.2 mmol/l of each dNTP, 1 μl of 10-μmol/l forward and reverse primers and 0.5 U of Taq polymerase in 1X PCR Buffer (GoTaq, Promega, Italy, Italy). The primers for the PCR reaction are reported in Table A (online supplemental data).

To detect the CYP2C19*2 polymorphism, 10 μl of the PCR products (321 bp) are subjected to digestion with SmaI restriction enzyme (GoTaq) and the reaction is carried out at 25°C for 4 h. To detect the IVS10 + 12G/A CYP3A4 polymorphism, 10 μl of the PCR products (222 bp) require an enzymatic digestion with Rsal (GoTaq) at 37°C for 4 h. The digestion fragments are separated on 3.5% agarose gel.

**Statistical analysis**

Statistical analysis was performed using the SPSS statistical package Version 11.5 (SPSS Inc., Chicago, Illinois, USA). At the posthoc power calculation for the association of the CYP2C19*2 rare allele with the 10-μmol/l ADP-RPR condition in our ACS population, our study has a β = 0.73. We tested whether the allele frequencies conformed to Hardy–Weinberg equilibrium proportions by the χ2 test. Genotype and allele frequencies were compared between groups by χ2 analyses. Categorical variables are expressed as frequencies and percentages. Unless otherwise indicated, data are given as median values and range. Comparisons of continuous variables between patients with and without RPR or among genotypes were performed by using the nonparametric Mann–Whitney U test or the analysis of variance (Kruskal–Wallis). To test the association of the CYP2C19*2 polymorphism with the occurrence of higher platelet reactivity, we applied the univariate linear regression analysis model with the 2-μmol/l ADP, 10-μmol/l ADP and log (AA) platelet aggregation analyzed as continuous variables, and the multivariate linear regression model with 2-mmol/l ADP or 10-mmol/l ADP or log (AA) platelet aggregation as dependent variables and age, sex, hypertension, diabetes mellitus, dyslipidemia and smoking habit as independent variables. A value of P < 0.05 was chosen as the cutoff level for statistical significance.

**Results**

In Table 1, demographic and clinical characteristics of patients are reported.

The P2Y12 T744C, CYP3A4 IVS10 + 12G/A and CYP2C19*2 polymorphisms were in Hardy–Weinberg equilibrium.

The genotype distributions of the three polymorphisms in the overall patient population are shown in Tables 2 and 3. The rare allele frequencies of the P2Y12 T744C, CYP3A4 IVS10 + 12G/A and CYP2C19*2 polymorphisms were 0.104, 0.116 and 0.171, respectively.
In patients with 10-µmol/l ADP-RPR, diabetes was more prevalent (32.0%) compared with patients without 10-µmol/l ADP-RPR (19.1%; \( P < 0.0001 \)). Moreover, patients with 10-µmol/l ADP-RPR were significantly \( (P = 0.012) \) older (median age 71 years, range 47–93) compared with patients without 10-µmol/l ADP-RPR (median age 69 years, range 27–94). No differences were observed between patients with and without 10-µmol/l ADP-RPR for the other clinical, demographic and therapeutic characteristics. In patients with AA-RPR, diabetes was more prevalent (27.6%) than in patients without AA-RPR (19.1%; \( P = 0.006 \)). Moreover, patients with AA-RPR were significantly \( (P < 0.0001) \) older (median age 72 years, range 27–93) compared with patients without AA-RPR (median age 68 years, range 34–94). No differences were observed between patients with and without AA-RPR for the other clinical, demographic and therapeutic characteristics. In patients with 10-µmol/l ADP-RPR, a higher prevalence of individuals with AA-RPR (56.9%) compared with patients without 10-µmol/l ADP-RPR (16.8%; \( P < 0.0001 \)) was observed. Patients receiving proton pump inhibitor treatment were >95%.

The P2Y12 T744C, CYP3A4 IVS10 + 12G/A and CYP2C19*2 polymorphisms and platelet function by platelet-rich plasma aggregation

Platelet aggregation after 2 and 10 µmol/l ADP and AA stimuli varied in the patient population according to the CYP2C19*2 polymorphism genotypes (Table 2). Carriers of the rare allele \(*1/*2 + *2/*2\) genotypes of the CYP2C19 polymorphism had significantly higher platelet aggregation values than noncarriers of the rare allele \(*1/*1\) after 2 µmol/l and 10 µmol/l ADP, and AA stimuli (Table 2). In particular, homozygous individuals for the rare allele had significantly higher platelet aggregation after 2 and 10 µmol/l ADP stimuli than heterozygous carriers (Table 2).

No differences in platelet aggregation values after the three different stimuli in the studied population according to the P2Y12 T744C and CYP3A4 IVS10 + 12G/A genotypes were observed (Table 3).

The analysis of the effect of the genotype combinations of the three polymorphisms did not evidence an interaction in inducing higher platelet aggregation values (data not shown).

The P2Y12 T744C, CYP3A4 IVS10 + 12G/A and CYP2C19*2 polymorphisms and antiplatelet drug response

Of the 1419 enrolled patients, 255 (18.0%) showed 10-µmol/l ADP-RPR. The genotype distribution of the CYP2C19*2 polymorphism significantly differed between patients with and without 10-µmol/l ADP-RPR \( (P = 0.002; \text{Fig. 1a}) \).

The genotype distribution of the P2Y12 and CYP3A4 polymorphisms was similar in patients with and without 10-µmol/l ADP-RPR (data not shown).

Of the 1419 enrolled patients, 341 (24.0%) showed AA-RPR. The genotype distribution of the CYP2C19*2 polymorphism significantly differed between patients with and without AA-RPR \( (P = 0.045; \text{Fig. 1b}) \).

The genotype distribution of the P2Y12 and CYP3A4 polymorphisms was similar in patients with and without AA-RPR (data not shown).

In Fig. 2, the genotype distribution according to the CYP2C19 genotypes in patients without any kind of RPR, with dual 10-µmol/l ADP-RPR + AA-RPR, with isolated AA-RPR or isolated 10-µmol/l ADP-RPR is reported. A statistically significant difference with respect to the

---

### Table 1: Demographic and clinical characteristics of patients

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Hypertension N (%)</th>
<th>Smoking habit N (%)</th>
<th>Dyslipidemia N (%)</th>
<th>Diabetes N (%)</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>69 (27–94)</em></td>
<td>1036/383</td>
<td>922 (65.0%)</td>
<td>581 (40.9%)</td>
<td>796 (56.1%)</td>
<td>299 (21.1%)</td>
<td><em>875 (61.7%)</em></td>
</tr>
<tr>
<td>10-µmol/l ADP-RPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1233 (86.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-inhibitors N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1168 (82.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme.

---

### Table 2: Distribution of maximal platelet aggregation after different stimuli in the overall study population according to CYP2C19*2 genotypes

<table>
<thead>
<tr>
<th>CYP2C19*2 genotypes</th>
<th>*1/*1</th>
<th>*1/*2</th>
<th>*2/*2</th>
<th>( P ) (overall)</th>
<th>*1/*2 + *2/*2</th>
<th>( P ) (vs. *1/*1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants N (%)</td>
<td>974 (68.6%)</td>
<td>405 (28.6%)</td>
<td>40 (2.8%)</td>
<td>445 (31.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggregation according to stimulus %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (0 µmol/l)</td>
<td>26 (1–100)</td>
<td>32 (1–94)*</td>
<td>41 (5–84)*</td>
<td>&lt;0.0001</td>
<td>33 (1–94)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ADP (10 µmol/l)</td>
<td>49 (1–100)</td>
<td>54 (2–100)*</td>
<td>62 (26–100)*#</td>
<td>&lt;0.0001</td>
<td>56 (2–100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Arachidonic acid (0.5 µmol/l)</td>
<td>11 (1–100)</td>
<td>12 (1–100)</td>
<td>14 (5–85)</td>
<td>0.060</td>
<td>12 (1–100)</td>
<td>0.043</td>
</tr>
</tbody>
</table>

ADP, adenosine 5'-diphosphate.

\*\( P < 0.0001 \) vs. *1/*1.

\#\( P = 0.028 \) vs. *1/*2.

\#\( P = 0.015 \) vs. *1/*2.
group of patients without any kind of RPR was observed in 10-μmol/l ADP-RPR ($P = 0.048$) and dual 10-μmol/l ADP-RPR + AA-RPR ($P = 0.015$).

**Univariate and multivariate linear regression analysis**

The univariate linear regression analysis model with the 2-μmol/l ADP, 10-μmol/l ADP and log (AA) platelet aggregation analyzed as continuous variables showed that the CYP2C19*2 allele was significantly associated with antiplatelet treatment variability (2-μmol/l ADP aggregation levels $\beta = 5.3$, SE = 1.0, $P < 0.0001$, $r = 0.142$; 10-μmol/l ADP aggregation levels $\beta = 5.8$, SE = 1.2, $P < 0.0001$, $r = 0.143$; log-transformed AA aggregation levels $\beta = 0.13$, SE = 0.04, $P < 0.0001$, $r = 0.087$). At the multivariate linear regression model with 2-μmol/l ADP or 10-μmol/l ADP or log (AA) platelet aggregation as dependent variables and age, sex, hypertension, diabetes mellitus, dyslipidemia and smoking habit as independent variables, age, diabetes and the CYP2C19*2 polymorphism remained significant and independent risk factors for antiplatelet treatment variability (Table 4).

**Discussion**

In this study we investigated, for the first time, the role of the CYP2C19*2 loss-of-function polymorphism, and of the
IVS10 + 12G/A CYP3A4 and T744C P2Y12 polymorphisms in modulating platelet reactivity in high-risk vascular patients treated with combined clopidogrel and aspirin therapy. This study provides the novel finding that the 2C19*2 allele of the CYP2C19 gene is an independent risk factor for higher platelet aggregability on antiplatelet treatment in high-risk vascular patients. This result strengthens the preliminary observation, in 28 healthy individuals who were administered clopidogrel [24], that the *2 allele of CYP2C19 is associated with higher platelet reactivity. Moreover, this study shows, on a large ACS population, that two other common polymorphisms, the IVS10 + 12G/A in the CYP3A4 gene and the T744C in the P2Y12 gene, are not related to higher platelet aggregability and to the RPR in patients on dual antiplatelet treatment.

Recently, the CYP2C19*2 allelic variant, encoding a deficient drug-metabolizing enzyme CYP2C19 [26], was demonstrated to be associated with an impaired responsiveness to a 7-day oral course of 75 mg/day of clopidogrel in 28 young healthy white men, as assessed ex vivo in terms of 10-μmol/l ADP platelet aggregation [24]. This influence was confirmed by measuring vasodilator-stimulated phosphoprotein (VASP) phosphorylation before and after clopidogrel administration [24]. The study of Hulot et al. [24] was in keeping with preliminary data in healthy individuals showing that the CYP2C19*2 polymorphism reduces 20-μmol/l ADP-induced platelet aggregation four and 24 h after a single oral dose of 300 mg of clopidogrel [25]. Our data obtained after 600 mg/day of clopidogrel suggest that the CYP2C19*2 polymorphism effect is not overcome by the high clopidogrel loading dose.

Our data show that the presence of the CYP2C19*2 polymorphism, but not of the CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with

### Table 4 Linear regression analysis with ADP 2 μmol/l or ADP 10 μmol/l or log (AA) platelet aggregation as dependent variable and age, sex, hypertension, diabetes mellitus, dyslipidemia and smoking habit as independent variables

<table>
<thead>
<tr>
<th></th>
<th>ADP 2 μmol/l</th>
<th></th>
<th>ADP 10 μmol/l</th>
<th></th>
<th>Log (AA)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β (SE)</td>
<td>P</td>
<td>β (SE)</td>
<td>P</td>
<td>β (SE)</td>
<td>P</td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>4.9 (1.3)</td>
<td>0.0001</td>
<td>5.5 (1.3)</td>
<td>0.0001</td>
<td>0.13 (0.06)</td>
<td>0.013</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6.7 (1.5)</td>
<td>0.0001</td>
<td>6.6 (1.7)</td>
<td>0.0001</td>
<td>0.22 (0.07)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.3 (0.06)</td>
<td>0.0001</td>
<td>0.3 (0.07)</td>
<td>0.0001</td>
<td>0.02 (0.003)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

AA, arachidonic acid; ADP, adenosine 5’ diphosphate.

Fig. 2

= Without RPR
| With isolated 10 μM ADP-RPR
| With isolated AA-RPR
| With combined 10 μM ADP-RPR and AA-RPR

Genotype distribution of the CYP2C19*2 polymorphism according to isolated AA-RPR, isolated 10-μmol/l ADP-RPR and dual 10-μmol/l ADP-RPR + AA-RPR. AA, arachidonic acid; ADP, adenosine 5’ diphosphate; RPR, residual platelet reactivity.
higher platelet reactivity, as evaluated by the PRP aggregation induced by 2 μmol/l and 10 μmol/l ADP, but also after a nonspecific clopidogrel agonist (AA). A great concern was voiced about the antiplatelet response in *2/*2 CYP2C19 homozygotes. This, to the best of our knowledge, has not yet been reported owing to the few numbers of individuals enrolled in the two previous studies aimed at evaluating the effect of this polymorphism on antiplatelet response in healthy persons. Our data demonstrated that homozygous individuals for the rare *2 allele had significantly higher platelet aggregation after 2-μmol/l and 10-μmol/l ADP stimuli than heterozygous carriers. The observation that a polymorphism involved in the metabolism of clopidogrel could influence platelet reactivity tested by both ADP and AA stimuli should not surprise us. Assessment of PRP aggregation by using the optical aggregometer, although induced by a specific agonist, is a global test that is influenced by the complex network of pathways that determine platelet reactivity. Platelet aggregability by 10 μmol/l ADP should be considered to be only partly independent of thromboxane A2 generation; therefore a polymorphism that causes a reduced responsiveness to clopidogrel might determine also a higher platelet aggregability by AA. Moreover, different mechanisms (such as other polymorphisms in the platelet membrane glycoproteins or enzymes, diabetes) could simultaneously cooperate with the platelet reactivity phenotype of each patient.

Our data show, for the first time, that the prevalence of the CYP2C19*2 polymorphism is significantly higher in patients with RPR than in patients without RPR defined according to AA-induced and 10-μmol/l ADP-induced platelet aggregation.

This study showed that the CYP2C19*2 allele accounted for about 2.0% of the variability of platelet reactivity at the univariate linear regression analysis, and that this association was independent after adjustment for classical risk factors. In fact, in the multivariate linear regression model, with 2-μmol/l ADP or 10-μmol/l ADP or log (AA) platelet aggregation as the dependent variable, age, diabetes and the CYP2C19*2 polymorphism remained significant and independent risk factors for higher platelet aggregability. The presence of each additional *2 rare allele determines an estimated increase in platelet aggregation by the 2-μmol/l and 10-μmol/l ADP of about 5%.

Our study was focused on patients admitted for ACS, in whom dual antiplatelet-treatment effectiveness was evaluated 24 h after a 600-mg clopidogrel and a 500-mg ASA loading dose. Owing to the major impact on the association of the CYP2C19*2 single nucleotide polymorphism and antiplatelet therapy responsiveness of diabetes and age, we cannot definitely exclude that ACS, known to affect platelet reactivity and antiplatelet responsiveness [31], might influence the results of the observed association and that the impact of this single nucleotide polymorphism might be different in stable cardiovascular patients treated with 75 mg/day of clopidogrel and 100 mg/day of ASA. In this study, we did not evaluate platelet function before PCI; nevertheless, different clinical studies demonstrated that post-PCI residual platelet reactivity (RPR) is an independent predictor of 1-year major adverse coronary events in patients with acute MI and of stent thrombosis in patients receiving drug-eluting stents [6,9,11–14,16,17].

Lau et al. [21] reported that CYP3A4 metabolic activity is associated with between-individual variability in clopidogrel responsiveness. A recent study, aimed at investigating the role of gene sequence variations of the CYP3A4 gene in modulating platelet activation in 82 stable CAD patients in a steady state (8 months) of treatment with combined clopidogrel and aspirin therapy. This study showed that carriers of the IVS10 + 12G/A polymorphism had reduced GPIIb/IIIa activation, as measured by assessing the platelet surface expression of the activated GpIIb/IIIa after a 2-μmol/l ADP stimulus through flow cytometry compared with noncarriers of the variant allele. In contrast, the polymorphism did not influence ADP–induced platelet aggregation profiles [27]; similar results were obtained also in clopidogrel-naive patients after the first 24-h treatment [27]. Our data demonstrate, on a large population of ACS patients on dual antiplatelet therapy, that the CYP3A4 IVS10 + 12G/A polymorphism is not associated with higher platelet aggregability and with residual platelet reactivity.

As concerns P2Y12 receptor gene, two haplotypes each composed of four polymorphisms in total linkage disequilibrium were identified, and the minor haplotype was found to be associated with increased in-vitro ADP-induced platelet aggregation [28]. Our results, demonstrating that the P2Y12 T744C polymorphism does not modulate platelet response to dual antiplatelet treatment, strengthen those obtained by other authors [29,30] on 597 patients receiving a 600-mg clopidogrel loading dose [30] and 83 patients on long-term clopidogrel (75 mg/day) treatment [29].

In conclusion, we have identified the CYP2C19*2 polymorphism as a determinant of higher platelet aggregability and residual platelet reactivity, which are related to an increase of clinical complications [17] in a high-risk population.

Further studies are required to investigate the influence of the contemporary presence of functional polymorphisms in the CYP2C19 and other genes involved in antiplatelet therapy responsiveness, as well as of other RPR determinants. The complex network of determinants should be studied keeping in mind their role in...
modulating platelet reactivity in relation to increasing doses or treatment with new antiplatelet drugs.

**Supplementary data**

Supplementary data are available at The FPC journal Online (www.pharmacogeneticsandgenomics.com).

**Acknowledgements**

This work was supported by grants from the Genpolis government FIRB project and from the Ente Cassa di Risparmio di Firenze to Fioren Foundation, Florence, Italy.

All authors disclaim any potential conflict of interest.

**References**