Influence of CYP450 enzymes, CES1, PON1, ABCB1 and P2RY12 polymorphisms on clopidogrel response in patients subjected to a percutaneous neurointervention procedure

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CONFLICT OF INTERESTS

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INTRODUCTION

Clopidogrel is one of the most used agents in the perioperative management of patients undergoing neurointerventional procedures. It is a thienopyridine prodrug usually given in combination with acetylsalicylic acid to prevent atherothrombotic and thromboembolic events (1).

Clopidogrel absorption is mainly limited by P-glycoprotein (P-gp) (2), encoded by *ABCB1*, an ATP-dependent transporter located in the intestinal epithelial cell wall which expels the drug into the intestinal lumen. Afterwards, clopidogrel is extensively metabolized in the liver. Most of the parent drug (approximately 85%) is metabolized to its inactive form (carboxylic acid) by carboxylesterase 1 (CES1) (3). The remaining (15%) suffers two sequential oxidative stages, through several cytochrome P450 (CYP) enzymes, originating the active metabolite. First, CYP2C19, CYP2B6 and CYP1A2 isoforms convert clopidogrel to 2-oxo-clopidogrel. Second, CYP3A4, CYP3A5, CYP2B6, CYP2C9 and CYP2C19 isoforms and the enzyme paraoxonase-1 (PON1) transform 2-oxo-clopidogrel into its active form (4,5). Besides, 50% of the formed 2-oxo-clopidogrel is also metabolized by CES1 to an inactive compound, consequently, limiting the amount of active metabolite (6). The active metabolite contains a thiol group which binds irreversibly to platelet $P2Y_{12}$ receptors, thereby inhibiting platelet activation and aggregation (7).

Several studies have evaluated the influence of CYP2C19 polymorphisms on clopidogrel effect. The presence of CYP2C19 intermediate metabolizer (IM) and poor metabolizer (PM) phenotypes has been associated with a hyporesponsiveness to clopidogrel, since they show lower levels of the active metabolite. Therefore, carriers of these variants have a higher risk of recurrent vascular events (8–11). On the other hand, carriers of CYP2C19 ultra-rapid metabolizer (UM) phenotype could show greater platelet inhibition and a hyperresponsiveness to clopidogrel (12–15), and consequently, an increased risk of hemorrhagic complications (10,16,17). With this respect, the Clinical Pharmacogenetics Implementation Consortium (CPIC) made a series of therapeutic recommendations based on CYP2C19 genotype for the treatment of acute coronary syndromes with clopidogrel (18).

Regarding neurovascular conditions, there is no genotype-guided therapeutic recommendation. Our group has previously described an association between the IM-PM phenotype and a hyporesponse to clopidogrel, along with a significantly higher rate of hemorrhagic events in UM patients undergoing a percutaneous neurointervention (19). In the

current study, our aim is to evaluate the effect of other genes involved in clopidogrel absorption and metabolism in this cohort of patients, including 21 new cases. This knowledge is of great importance to ensure the correct antiaggregation of these patients and to avoid as far as possible the risk of subsequent events.

RESULTS

Patient characteristics

Our study population comprised 144 patients (74 men and 70 women). Table 1 shows their main demographic characteristics. 46.5% of the patients were intervened due to the presence of an aneurysm and 51.4% presented a stenosis. Aneurysms were more frequently found in women; while stenosis was more frequently detected in men.

The genotype frequencies are shown in supplementary table 2. There was no difference in the distribution of genotype frequencies among sexes, except for *CYP1A2**1C in which all the carriers were women (p=0.025), and *CYP1A2**1B in which more women carried the *1/*1 genotype (32.4%) than men (13.9%), p=0.034.

Patient outcome

The mean aggregation value (measured in 141 of the patients) was 161.3± 87.3 PRU. Men showed higher aggregation value (173.8± 86.0 PRU) than women (148.1 ± 87.3 PRU), although it was not significant (p=0.081) (table 1). According to this parameter, 56% of the patients were categorized into responders, being this percentage higher in women (64%) than in men (49%), p=0.090. In all, 5% of the patients required a dose reduction (8.6% of women and 1.4% of men, p=0.058), while 14% required a change of treatment. The median treatment duration was 80 days, with a range of 1-3079 days.

Regarding the primary outcome, 18.8% of the patients experienced a clinical event. The incidence of ischemic events was higher (10.4%) than the incidence of hemorrhagic events (8.3%), with no significantly different distribution among sexes. During the neurointerventional procedures, 4 patients experienced a hemorrhage due to a perforation of an artery. Two of them were CYP2C19 IM-PM, one was CYP2C19 NM and another one was CYP2C19 UM. These hemorrhages were not considered in the analysis since they were not related to clopidogrel

treatment but related to the procedure. Table 2 shows a summary of the patients' outcome according to the different genotypes/phenotypes of the genes analyzed.

Influence of CYP2C19 on patient outcome

CYP2C19 clearly had an influence on the patients' outcome. *CYP2C19* IM-PM patients showed a significantly higher aggregation value, which led to a significant worse response to clopidogrel (table 2). This lack of response may explain a significant shorter treatment duration (non-standardized β coefficient = -235.6; p = 0.027) in these patients.

Moreover, regarding the primary outcome, the incidence of ischemic events was lower in the UM group (2.3%) compared to IM-PM (10.8%) and NM (15.9%), p=0.060. Figure 1a shows the time until appearance of an ischemic event, with a significant difference between the survival functions of the three phenotypes (p=0.043). The comparison by pairs with Statistical Log-Rank did not detect significant differences in IM-PM compared to NM (p=0.996) and IM-PM compared to UM (p=0.076); while there was a difference in UM compared to NM (p=0.017). Moreover, Haberman-corrected typed residues pointed to a significant lower frequency of ischemic events in the UM group (2.3%) than the expected under the hypothesis of independence between variables (9.7%); while the frequency of ischemic events in NM (15.9%) was not significantly higher compared to the expected.

The highest incidence of hemorrhagic events was detected in the UM group (15.9%) compared to NM (6.3%) and IM-PM (2.7%), although this difference did not reach statistical significance (p=0.101). The hemorrhagic events onset time is shown in figure 1b, showing a difference between the survival functions of the three phenotypes (p=0.041). However, the comparison by pairs with statistical log-rank did not detect significant differences: IM-PM compared to NM p=0.547; IM-PM compared to UM: p=0.078; NM compared to UM: p=0.097. Nevertheless, Haberman-corrected typed residues showed a significantly higher frequency of hemorrhagic events in the UM group (15.9%) than expected (8.3%); while the frequencies observed in the IM-PM and NM groups did not differ significantly from the expected under the hypothesis of independence between variables.

Influence of other CYP enzymes, CES1 and PON1 enzymes on patient outcome

There was no influence of CYP2C9, *CYP2C8*, CYP1A2, CYP2B6, *CYP3A4* and *CYP3A5* on the aggregation value (table 2).

Concerning *CES1*, although it did not reach statistical significance, we observed that patients carrying the C/T genotype of rs71647871 showed a considerable lower aggregation value (59.0 ± 21.2 PRU) compared to the wild-type genotype (165.2 ± 86.0 PRU), p=0.084. There was no influence of PON1 enzyme on the aggregation value.

Regarding the primary outcome, the incidence of ischemic events was higher in the *CYP2C9* PM-IM phenotype (15.9%) compared to NM (5.6%), but it was not statistically significant (p=0.059). Nevertheless, neither other CYP enzyme, CES1 nor PON1 had a significant influence on the incidence of ischemic or hemorrhagic subsequent events.

Influence of ABCB1 on patient outcome

There was a tendency towards a lower aggregation value in patients carrying the mutated alleles of *ABCB1* C3435T, C1236T and G2677T/A. In fact, the percentage of responders was significantly higher in patients carrying the mutated haplotype (table 2). However, there was no association between *ABCB1* haplotypes and the incidence of ischemic or hemorrhagic events.

Influence of P2RY12 on patient outcome

Neither individual polymorphisms nor *P2RY12* haplotypes H1 and H2, including rs10935838, rs2046934, rs5853517 and rs6809699, had no influence on the aggregation value. Likewise, there was no association between *P2RY12* polymorphisms and the incidence of ischemic or hemorrhagic events.

Influence of the concomitant treatment with proton-pump inhibitors

In all, 76.4% of the patients were receiving proton-pump inhibitors (PPIs) as a concomitant treatment, which are CYP2C19 inhibitors. Of them, 50% were receiving omeprazole and 50% pantoprazole. Patients under PPIs treatment showed a significantly higher aggregation value (170.7 ± 84.5 PRU) compared to those without PPIs treatment (129.0 ± 90.2 PRU), p=0.017. Moreover, both patients receiving omeprazole and pantoprazole showed similar aggregation value (170.8 ± 84.1 PRU and 170.6 ± 85.8 PRU, respectively). However, there was no influence of the concomitant treatment with PPIs on the incidence of both ischemic (10.9% of patients receiving PPIs vs. 8.8% of patients not receiving PPIs, p=0.768) and hemorrhagic events (9.1% of patients receiving PPIs vs. 5.9% of patients not receiving PPIs, p=0.732).

Results from the multivariate analysis

A multiple regression analysis was performed considering the aggregation value, the response rate, the incidence of ischemic and hemorrhagic events as dependent variables. It included sex, age, all the genes analyzed, presence of cardiovascular risk factor (hypertension, dyslipidemia, obesity, atrial fibrillation, diabetes mellitus, current smoker), previous ischemic of hemorrhagic events, type of intervention and concomitant treatment with PPIs as independent variables. A summary of the results is shown in table 3.

Briefly, age, PPIs concomitant treatment and *CYP2C19* IM-PM phenotype appeared to be predictors of a worse response due to a higher aggregation value. Besides, being intervened with a flow diverter was *a* predictor of a better response, compared to stent and coils intervention. This four factors explained the 26.4% of the model variance (r^2=0.327). When transforming the aggregation value into a categorical variable, we observed that age and *CYP2C19* IM-PM continued to be worse response predictors. Additionally, *ABCB1* mutated haplotype appeared as a predictor of a better response (r^2=0.433).

In addition, as shown in table 3, *CYP2C19* UM phenotype was a protective factor while the treatment duration was a risk factor for the development of ischemia. Regarding the prediction of hemorrhagic events, CYP2C19 UM appeared to be the only risk factor.

DISCUSSION

The variability related to clopidogrel response is a well-known aspect, since a range of 4-30% of the patients are non-responders (20). Therefore, these patients are at increased risk of ischemic events after stent implantation (21). Factors such as age, body mass index, comorbidities, concomitant treatment, and compliance explain less than 10% of this variability (22). Consequently, the role genetics play could be of great importance.

Influence of CYP2C19

According to the expected, we found that *CYP2C19* UM phenotype is a protective factor for the development of ischemic events, which was already observed in our previous work (23). Our results contradict those of Lin *et al.,* who found that carriage of *CYP2C19**17 allele was associated with the incidence of ischemic events (24). In fact, we already showed an increased risk of bleeding in *CYP2C19* UM patients (19,23), which Haberman-corrected typed residues confirmed in the current study with a larger sample size. We encourage considering this fact for the treatment approach of these patients.

Furthermore, Zhu *et al.* established a correlation between *CYP2C19* no function alleles and an increased risk of subsequent ischemic events in patients subjected to a stent implantation in the carotid artery (25). However, in our study, we could not confirm this fact, probably due to the small sample size. Moreover, carriers of *CYP2C19**2 allele are more closely evaluated. In fact, after the vast amount of evidence, most clinicians decide to change clopidogrel for an alternative therapy to avoid the risk of subsequent events. Conversely, what we could confirm is the premise by Colley and Yan, who published a revision about the association between the carriage of *CYP2C19**2 allele and a hyporesponse to clopidogrel in neurointervened patients (1). This circumstance was already observed in our previous work with a lower sample size (19).

Besides, Moore *et al.* compared the efficacy, safety and cost of the treatment with clopidogrel *vs*. ticagrelor in patients with cerebral aneurysms treated with flow diverter. They found that ticagrelor was not inferior when preventing thromboembolic complications. However, due to the much higher costs of ticagrelor, this alternative therapy should be used only in clopidogrel non-responders (26). Based on our results, we suggest that both *CYP2C19* IM-PM and UM should be given an alternative antiplatelet therapy.

Influence of other CYP enzymes, CES1 and PON1

There is controversy whether there is an association of *CYP2C9* most studied alleles (*2 and *3) and clopidogrel effect. Some authors state no significant relationship (12) while others associate the presence of *3 allele with a higher incidence of stent thrombosis (27). In our study *CYP2C8* or *CYP2C9* were not associated with a difference in clopidogrel response. However, we observed a tendency towards a higher incidence of ischemic events in subjects carrying *CYP2C8* or *CYP2C9* PM- IM phenotype, which was not statistically significant neither in univariate nor multivariate analyses. Further approaches with larger sample sizes would be of interest.

Reduced CYP3A4 activity has been associated with an increased risk of stent thrombosis in patients with acute coronary syndrome treated with clopidogrel (28). Indeed, one study postulates that the role of CYP3A4/5 in the metabolism of clopidogrel may be of greater relevance than has been previously described (29). However, in our study, CYP3A4 and CYP3A5 did not show a significant role in explaining some of the response variability. Our results resembled those of Holmberg *et al.* who found that neither CYP3A4 nor CYP3A5 genotypes

affected clopidogrel area under the concentration-time curve or platelet inhibition in healthy volunteers (30).

Furthermore, the G143E polymorphism (rs71647871) described in *CES1* was associated with a decreased protein functionality (31). Lewis *et al*. found that carriers of the mutated allele showed higher levels of the active metabolite and, therefore, a better response to clopidogrel in patients with coronary disease (32). Consistent with these studies, we have found a tendency towards a lower aggregation value in patients carrying the mutation, which can be explained by an increased active metabolite formation due to a lower CES1 functionality. However, our limited sample size was not sufficient to find statistically significant results, since we could only find two carriers of the G143E polymorphism. Further research is needed in this cohort of patients to confirm if there is an association.

Regarding *PON1*, it has been described that the Q192R polymorphism (rs662) conditions the active metabolite formation (5). In our study, we observed that patients carrying defective *PON1* alleles, assigned as PM and IM, showed a tendency towards a higher aggregation value, although it was far from significance. This fact is consistent with the previously reported by Verschuren *et al*, who found that patients carrying the defective allele of rs662 may have lower levels of the active metabolite, thus resulting in a poorer response and an increased risk of ischemic events (33). In our study, the incidence of ischemic events was higher in the PM group. Further research is warranted.

Influence of ABCB1

Taubert *et al*. described lower levels of clopidogrel and its metabolite in patients carrying the *ABCB1* C3435T T/T genotype, probably due to an increased expression of P-gp (2). Conversely, our results suggest that patients carrying the C3435T, C1236T or G2677T/A minor alleles have a reduced P-gp expression, since we found a better response prediction in patients carrying the *ABCB1* mutated haplotype. This would be explained by higher concentrations of clopidogrel and its metabolite, since the efflux pump would be working inefficiently. Some studies relate the most studied *ABCB1* polymorphism, C3435T, to a lower P-gp expression in minor allele carriers (34–37). Indeed, if the minor alleles are associated with reduced transporter functionality, it is expected that these patients show higher concentrations of P-gp substrate drugs, as a result of a minor elimination. For this reason, clopidogrel absorption might be influenced by *ABCB1* polymorphisms.

Influence of P2RY12

Finally, as *P2RY12* is the gene encoding for clopidogrel target receptor P2Y12, some polymorphisms (rs10935838, rs2046934, rs5853517 and rs6809699) have been associated with enhanced platelet reactivity (38,39). However, these associations were not replicated and the level of evidence is low. In our study, we could not find a significant association in *P2RY12* haplotypes related to clopidogrel response. The lack of association between P2RY12 polymorphisms and clopidogrel response match the results from Giusti *et al.* (9) and Cuisset *et al.* (40). They demonstrated that *P2RY12* rs2046934 polymorphism was not associated with antiplatelet activity in patients with acute coronary syndrome treated with clopidogrel. Moreover, Simon *et al.* described no association between rs16846673, rs6809699 and rs6785930 and risk of adverse cardiovascular events in patients with acute myocardial infarction receiving clopidogrel (41).

STUDY LIMITATIONS

Our main limitation is our unfeasibility of measuring clopidogrel and its active metabolite concentrations, which could have been useful to correlate it with patients' aggregation value and clinical outcome. Moreover, the small sample size limited us from finding more patients carrying some minor alleles with a low frequency that might be related to clopidogrel metabolism, like *CES1* polymorphisms. Hence, further investigation is warranted.

CONCLUSION

We confirmed that CYP2C19 is the most important enzyme involved in clopidogrel response. Indeed, the carriage of *CYP2C19**2 allele is strongly associated with a hyporesponse to clopidogrel in neurointervened patients. Carrying the *CYP2C19**17 allele is a protective factor for the development of ischemic events, while it is a risk factor for bleeding complications. An alternative therapy should be prescribed for *CYP2C19**2 carriers but also for patients carrying *CYP2C19**17 allele, to avoid bleeding complications. Moreover, we found a lower aggregation value in *ABCB1* mutated patients, being this haplotype a predictor of a better response, finding clopidogrel absorption clearly influenced by P-glycoprotein. Patients carrying the CES1 G143E C/T genotype showed a considerable lower aggregation value, although not significant, which suggest an increased active metabolite formation. To date, the influence of polymorphisms in other *CYP* enzymes, *CES1*, *PON1* or *P2RY12* is not demonstrated in patients subjected to neurointervention procedures, further research is needed.

MATERIALS AND METHODS

Study population, design and procedures

This retrospective observational study analyzed the clinical data of patients subjected to percutaneous neurointervention who were treated with clopidogrel. We included 144 patients from May 2013 to October 2018. The primary safety endpoint was the incidence of either thrombotic or hemorrhagic events during the treatment with clopidogrel, which could vary from a few days to several months. Other endpoints included: antiplatelet response, requirement of dose reduction, change of the antiplatelet therapy and treatment duration. All the data was collected from the medical records and included demographic factors (age and sex), cardiovascular risk factors (such as smoking status, hypertension, dyslipidemia, obesity, diabetes mellitus, chronic obstructive pulmonary disease, atrial fibrillation, acute myocardial infarction or previous ischemic and hemorrhagic events), and type of intervention. Concomitant treatment with CYP2C19 inhibitors was also taken into account.

This study complied with Declaration of Helsinki and current Spanish legislation on clinical research in humans and was approved by the Ethics Committee of Drug Research of Hospital Universitario de La Princesa.

Antiplatelet response

Antiplatelet response was documented with the VerifyNow System (Accriva Diagnostics, San Diego, CA), which determine the level of platelet P2Y12 receptor blockade (PRU - P2Y12 Reaction Unit) by determining the adenosine diphosphate (ADP) induced aggregation (extent of platelet aggregation in the presence of P2Y12 inhibitors). Platelet reactivity tests were performed prior to the intervention was accomplished. Values below 180 PRU suggest evidence of a P2Y12 inhibitor effect while values over 180 PRU suggest that there is no drug effect due to low P2Y12 inhibition response. Based on these values we classified the patients into responders to clopidogrel (values below 180 PRU) and non-responders (values over 180 PRU), according to the Verify Now Reference Guide (Accriva Diagnostics, San Diego, CA). Clopidogrel dose was adjusted according to hyper- or hypotreatment response.

Genotyping

DNA was extracted from 1 mL of peripheral blood samples using MagNA Pure LC DNA Isolation Kit in an automatic DNA extractor (MagNa Pure® System, Roche Applied Science, Indianapolis, Indiana) and quantified spectrophotometrically in a NanoDrop® ND-1000 Spectrophotometer

(NanoDrop Technologies Inc, Wilmington, Delaware), the purity of the samples was measured by the $A_{260}/_{280}$ absorbance ratio.

We analyzed 34 polymorphisms in 11 genes related to clopidogrel metabolism, transport and mechanism of action. A complete list of the analyzed variants and their functional consequences is described in Suplemmentary table 1.

CYP2C19*2, *3 and *17 genotyping was performed by real-time polymerase chain reaction (qPCR) with hybridization probes, designed and manufactured by TIB MOLBIOL (Berlín, Germany) in a LightCycler 2.0 device (Roche Bioscience, Mannheim, Germany), as previously described (19). Of the 144 samples genotyped for CYP2C19 variants, only 140 were available for genotyping the rest of the polymorphisms. *CYP1A2**1C (rs2069514), *CYP2B6**9 (rs3745274) and *PON1* rs705379 were genotyped by qPCR using a StepOne® PCR Instrument (Applied Biosystems, CA, USA) and TaqMan assays following the manufacturer recommendations (Applied Biosystems, CA, USA). The genotyping of *ABCB1, CYP1A2* (*1F and *1B*), CYP2B6*5, CYP2C8, CYP2C9, CYP3A4, CYP3A5, PON1, CES1, P2RY12, UGT1A1, UGT2B7* and *UGT2B4* was performed by MALDI-TOF mass spectrometry, with the MassARRAY® platform (Agena Bioscience Inc., San Diego, CA, USA). All assays were performed with an internal quality control, with a genotyping success rate of 100% and 100% reproducibility.

Statistical analysis

In order to simplify the analysis, genotypes were classified according to CPIC allele definition (42) and allele functionality tables, as the one described by our group for *CYP1A2* (43). Thus, *CYP2C19* genotypes were classified according to the number of functional alleles into IM-PM (*1/*2, *2/*2 and *2/*17), NM (*1/*1), and UM (*1/*17 and *17/*17). Moreover, *CYP2B6*, *CYP2C8* and *CYP2C9* genotypes were also classified into PM (carriers of two mutated alleles), IM (carriers of *CYP2B6**5 or *9, *CYP2C8* *2, *3 or *4 and *CYP2C9**2 or *3 in heterozygosis), and NM (carriers of *1/*1 genotype) according to the functionality of the alleles. *CYP1A2**1C, *1, *1B and*1F were assigned an activity score of 0.5, 1, 1.25 and 1.5, respectively. Thus, as previously validated (43), patients with *CYP1A2* *1/*1B, *1/*1F, *1B/*1B, *1C/*1F, *1C/*1B genotypes were categorized into *CYP1A2* NM/RM phenotype. Moreover, patients with *CYP1A2* *1B/*1F and *1F/*1F genotypes were assigned a UM phenotype. The only patient with *CYP1A2* *1C/*1C genotype was classified as PM. Additionally, patients with *CYP3A4* *1/*1 were classified as NM and patients carrying the *CYP3A4* *1/*22 and *22/*22 genotypes were classified as IM-PM. Likewise, patients with *CYP3A5* *1/*3 and *1/*6 genotypes were classified as “expressers” and patients carrying

CYP3A4 *3/*3 and *3/*6 genotype as “non-expressers”. Regarding PON1, as there is no functionality table that allows inferring a phenotype, we assigned the NM phenotype to those patients without any mutation. On the contrary, carriers of one mutated allele in any of the three variants analyzed (rs662, rs854560 and rs705379) were considered IM. Patients carriers of two mutated alleles in any of the three *PON1* variants were considered PM. Finally, ABCB1 C3435T, C1236T and G2677T/A genotypes were categorized into a haplotype “wild-type” when there was an absence of mutation, “heterozygote” when there was any heterozygous genotype in at least one of the three variants and “mutated” when there was two mutated alleles in any of the three polymorphisms.

For the analysis of the influence of all covariates, the aggregation value was translated into the variable “responders”, which classified agregometry values equal or higher than 180 PRU into non-responders patients and values lower than 180 PRU into responder patients.

Statistical analysis was performed using the SPSS 22.0 software (SPSS Inc., Chicago, Illinois); we considered p values lower than 0.05 to be statistically significant. Differences in genotype frequencies according to sex and the comparison of the qualitative variables between different genotypes were determined using a corrected Pearson chi-square test. Differences in quantitative parameters between individuals were statistically analyzed by a parametric univariate analysis (t test or ANOVA) or non-parametric univariate analysis (Kruskal-Wallis). A multiple regression analysis was performed to analyze the effect on aggregation value and incidence of posterior ischemic and hemorrhagic events. For this purpose, categorical variables with more than two categories, such as phenotype, were analyzed using dummy variables. The time until the appearance of an ischemic or hemorrhagic event has been studied by means of the survival analysis with the Kaplan-Meier procedure, and the differences between the groups were evaluated via the Log-rank test with linear trend for factor levels. Patients were censored when either clopidogrel treatment or the follow-up was finished.

STUDY HIGHLIGHTS

What is the current knowledge on the topic?

Although there is no standard guideline for clopidogrel treatment in patients with neurovascular conditions, the influence of *CYP2C19* alleles on clopidogrel response is already confirmed.

What question did this study address?

This is the first study to carry out a joint analysis of *CYP2C19* and other genes involved in clopidogrel treatment in patients subjected to percutaneous neurointervention.

What does this study add to our knowledge?

This study confirms that CYP2C19 is the most important enzyme involved in clopidogrel response. Indeed, *CYP2C19* UM phenotype is a protective factor for the development of ischemic events, while it is a risk factor for bleeding complications. In addition, *ABCB1* is a key factor affecting clopidogrel distribution. Finally, the presence of CES1 G143E polymorphism might increase clopidogrel response, which needs to be confirmed.

How this might change clinical pharmacology or translational science?

We encourage considering an alternative antiplatelet therapy in *CYP2C19* IM-PM and UM patients. Additionally, *ABCB1* polymorphisms could be considered for a better pharmacogenetic approach.

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AUTHOR CONTRIBUTIONS

M.S.R., C.B, D.K., P.Z. wrote the manuscript; J.L.C., E.B, D.O. and F.A.S designed the research; M.S.R, C.B., D.K, P.Z. and F.A.S. performed research, M.S.R. and D.R.P. analyzed the data; A.E. contributed with analytical tools.

Table 1. Main demographic and clinical characteristics of the study population.

	All (N=144)	Men (n=74)	Women (n=70)	P value
Age (mean ± SD)	64.8 ± 11.8	67.5 ± 11.5	61.9 ± 11.6	**0.004**
Presence of cardiovascular risk factors	129 (89.6)	70 (94.6)	59 (84.3)	0.056
Hypertension	91 (63.2)	52 (70.3)	39 (55.7)	0.085
Dyslipidemia	70 (48.6)	44 (59.5)	26 (37.1)	**0.008**
Obesity	3 (2.1)	1 (1.4)	2 (2.9)	0.612
Atrial fibrillation	3 (2.1)	3 (4.1)	0 (0)	0.245
Diabetes mellitus	28 (19.4)	19 (25.7)	9 (12.9)	0.060
Currently smoking	47 (32.6)	28 (37.8)	19 (27.1)	0.214
Previous ischemic and hemorrhagic events				
Acute myocardial infarction	9 (6.3)	8 (10.8)	1 (1.4)	**0.034**
Transient ischemic attack	16 (11.1)	15 (20.3)	1 (1.4)	**<0.001**
Ischemic stroke	20 (13.9)	14 (18.9)	6 (8.6)	0.093
Hemorrhagic stroke	11 (7.6)	1 (1.4)	10 (14.3)	**0.004**
Reason for intervention				
Aneurysms	67 (46.5)	15 (20.3)	52 (74.3)	**<0.001**
Stenosis	77 (53.5)	59 (79.7)	18 (25.7)	**<0.001**
Type of intervention				
Flow diverter	35 (24.3)	7 (9.5)	28 (40.0)	**<0.001**
Stent	94 (65.3)	63 (85.1)	31 (44.3)	
Coil	12 (8.3)	2 (2.7)	10 (14.3)	
No intervention	3 (2.1)	2 (2.7)	1 (1.4)	
Patients with concomitant treatment	141 (97.9)	73 (98.6)	68 (97.1)	0.612
OACs	4 (2.8)	4 (5.4)	0 (0)	0.120
ASA	139 (96.5)	73 (98.6)	66 (94.3)	0.200
Heparin	1 (0.7)	1 (1.4)	0 (0)	1.000
NSAIDs	7 (4.9)	2 (2.7)	5 (7.1)	0.266
PPIs	110 (76.4)	56 (75.7)	54 (77.1)	0.847
SSRIs	11 (7.6)	2 (2.7)	9 (12.9)	**0.028**
Patients outcome				
Aggregation value, PRU (n=141)	161.2 ± 87.3	173.8 ± 86.0	148.1 ± 87.3	0.081
Patients responding (n=141)[a]	79 (56.0)	35 (48.6)	44 (63.8)	0.090
Patients requiring dose reduction	7 (4.9)	1 (1.4)	6 (8.6)	0.058
Patients with change of treatment	20 (13.9)	7 (9.5)	13 (18.6)	0.149
Appearance of subsequent ischemic event	15 (10.4)	8 (10.8)	7 (10.0)	1.000
Appearance of subsequent hemorrhagic event	12 (8.3)	7 (9.5)	5 (7.1)	0.766

Values are presented as mean ± SD or n (%) and median (range) for treatment duration. Abbreviation: OACs, oral anticoagulants; ASA, acetyl salicylic acid; NSAIDs, non-steroid anti-inflammatory drug; PPIs, proton-pump inhibitors; SSRIs, selective serotonin reuptake inhibitors; PRU, P2Y12 Reaction Unit. [a]Patients were considered responders when PRU values were below 180.

Table 2. Outcome of patients undergoing cerebral vascular intervention according to the studied genes.

Gene	Genotype/Phenotype/ Haplotype	Aggregation value, PRU (n=141) [a, b]	Patients responding [c]	Ischemic events	Hemorrhagic events
CYP2C19	PM-IM (n=37)	200.1 ± 84.3	13 (35.1)	4 (10.8)	1 (2.7)
	NM (n=63)	140.3 ± 89.2	42 (67.7)	10 (15.9)	4 (6.3)
	UM (n=44)	157.9 ± 76.8	24 (57.1)	1 (2.3)	7 (15.9)
	p-value	0.004	0.007	0.06	0.101
CYP2C9	PM-IM (n=69)	153.7 ± 85.9	39 (56.5)	11 (15.9)	4 (5.8)
	NM (n=71)	173.8 ± 86.3	36 (52.9)	4 (5.6)	8 (11.3)
	p-value	0.173	0.733	0.059	0.367
CYP2C8	PM-IM (n=64)	150.7 ± 85.7	38 (59.4)	10 (15.6)	5 (7.8)
	NM (n=76)	175.1 ± 86.0	37 (50.7)	5 (6.6)	7 (9.2)
	p-value	0.100	0.390	0.104	1
CYP1A2	PM (n=1)	60	1 (100)	0 (0.0)	0 (0.0)
	NM/RM (n=70)	157.2 ± 86.0	41 (60.3)	7 (10.0)	7 (10.0)
	UM (n=69)	171.7 ± 86.5	33 (48.5)	8 (11.6)	5 87.2)
	p-value	0.303	0.201	0.814	0.784
CYP2B6	PM (n=17)	170.1 ± 65.3	11 (64.7)	3 (17.6)	1 (5.9)
	IM (n=61)	153.1 ± 82.9	33 (56.9)	7 (11.5)	4 (6.6)
	NM (n=62)	171.8 ± 94.4	31 (50.0)	5 (8.1)	7 (11.3)
	p-value	0.474	0.518	0.508	0.641
CYP3A4	NM (n=123)	165.7 ± 84.7	64 (52.9)	12 (9.8)	9 (7.3)
	PM-IM (n=17)	148.7 ± 99.1	11 (68.8)	3 (17.6)	3 (17.6)
	p-value	0.464	0.29	0.395	0.163
CYP3A5	Non-expressers (n=123)	179.6 ± 73.6	7 (41.2)	3 (17.6)	1 (5.9)
	Expressers (n=17)	161.4 ± 88.1	68 (56.7)	12 (9.8)	11 (8.9)
	p-value	0.418	0.3	0.395	1
CES1 rs71647871	C/C (n=138)	165.2 ± 86.0	71 (54.1)	15 (10.9)	12 (8.7)
	C/T (n=2)	59.0 ± 21.2	2 (100.0)	0 (0.0)	0 (0.0)
	p-value	0.084	0.501	1	1
PON1	PM (n=114)	165.3 ± 86.6	59 (52.7)	15 (13.2)	10 (8.8)
	IM (n=19)	164.1 ± 86.3	11 (57.9)	0 (0.0)	1 (5.3)
	NM (n=7)	130.2 ± 90.6	5 (83.3)	0 (0.0)	1 (14.3)
	p-value	0.626	0.358	0.184	0.673
ABCB1	Wild-type (n=30)	168.7 ± 87.0	13 (43.3)	4 (13.3)	2 (6.7)
	Heterozygous (n=83)	165.7 ± 88.5	41 (50.6)	10 (12.0)	8 (9.6)
	Mutated (n=27)	151.7 ± 80.9	21 (80.8)	1 (3.7)	2 (7.4)
	p-value	0.727	0.009	0.478	1
P2RY12	H1 (n=103)	167.2 ± 86.6	53 (53.0)	10 (9.7)	9 (8.7)
	H2 (n=4)	177.0 ± 78.1	2 (50.0)	0 (0.0)	0 (0.0)
	p-value	0.825	1	1	1

[a]Aggregation value was available for 141patients who were genotyped for *CYP2C19* and only in 137 patients evaluated for the rest of the genes. Values are expressed as n (%) but [b] mean ± SD. [c] Patients were considered responders when PRU values were below 180.

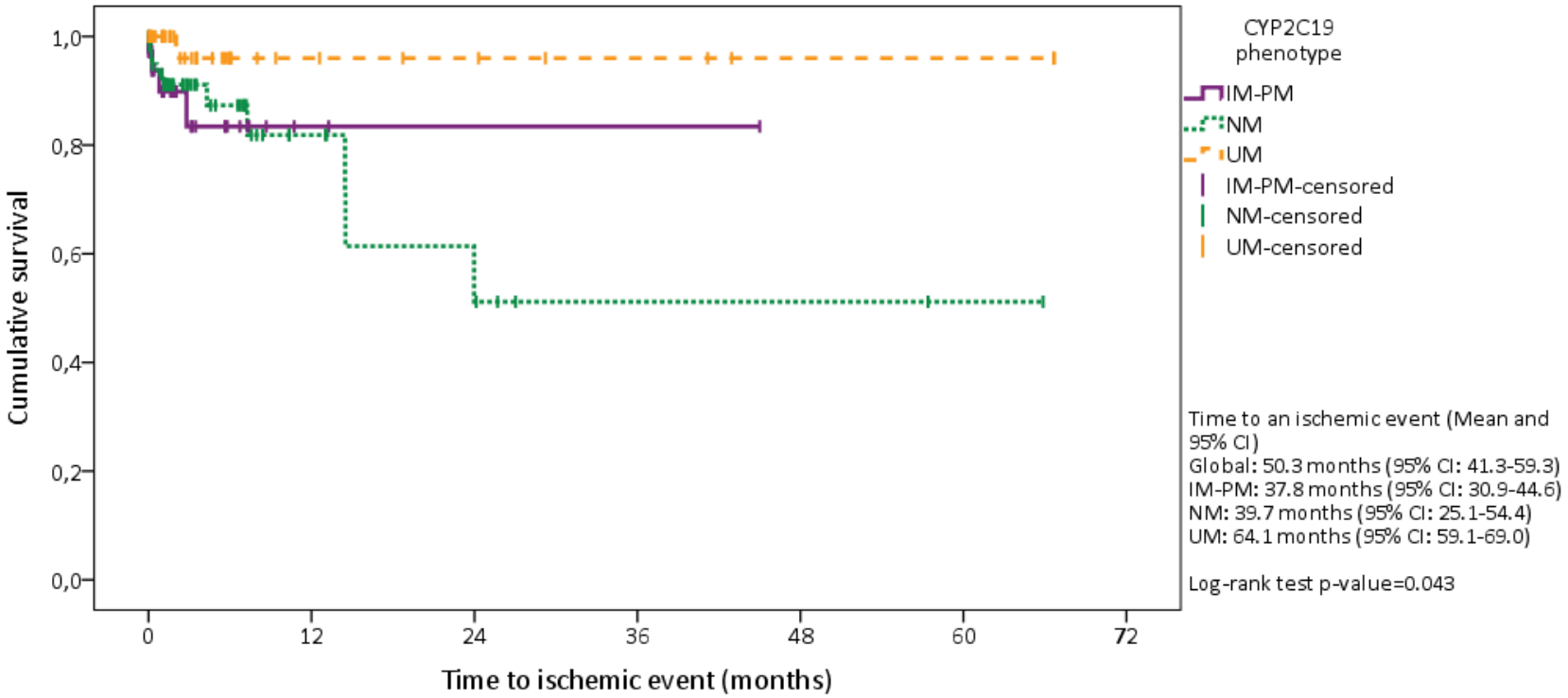
Table 3. Results from the multivariate analysis

Variables Contributing to P2Y12 Receptor Blockade (Aggregation value as dependent variable)						
Aggregation Value Predictors	Non-standardized β coefficient	p-value	Semi-partial correlation	Contribution to model variance	Additive contribution to model variance	P2Y12 Receptor Blockade
Age	2.235	0.001	0.288	8.2%	8.2%	Worse response
CYP2C19 IM-PM	59.519	<0.001	0.302	9.1%	17.3%	Worse response
Flow diverter	-52.848	0.004	-0.243	5.9%	23.2%	Better response
PPIs treatment	38.404	0.029	0.181	3.2%	26.4%	Worse response
Adjusted R-squared= 0.327						

Variables Contributing to P2Y12 Receptor Blockade (PRU>179 = Non-Responders)					
Response rate predictors	Odds-Ratio	Lower 95% CI	Upper 95% CI	p-value	P2Y12 Receptor Blockade
Age	0.895	0.849	0.943	<0.001	Worse response
CYP2C19 IM-PM	0.149	0.045	0.498	0.002	Worse response
ABCB1 mutated	6.298	1.555	25.499	0.010	Better response
R-squared= 0.433					

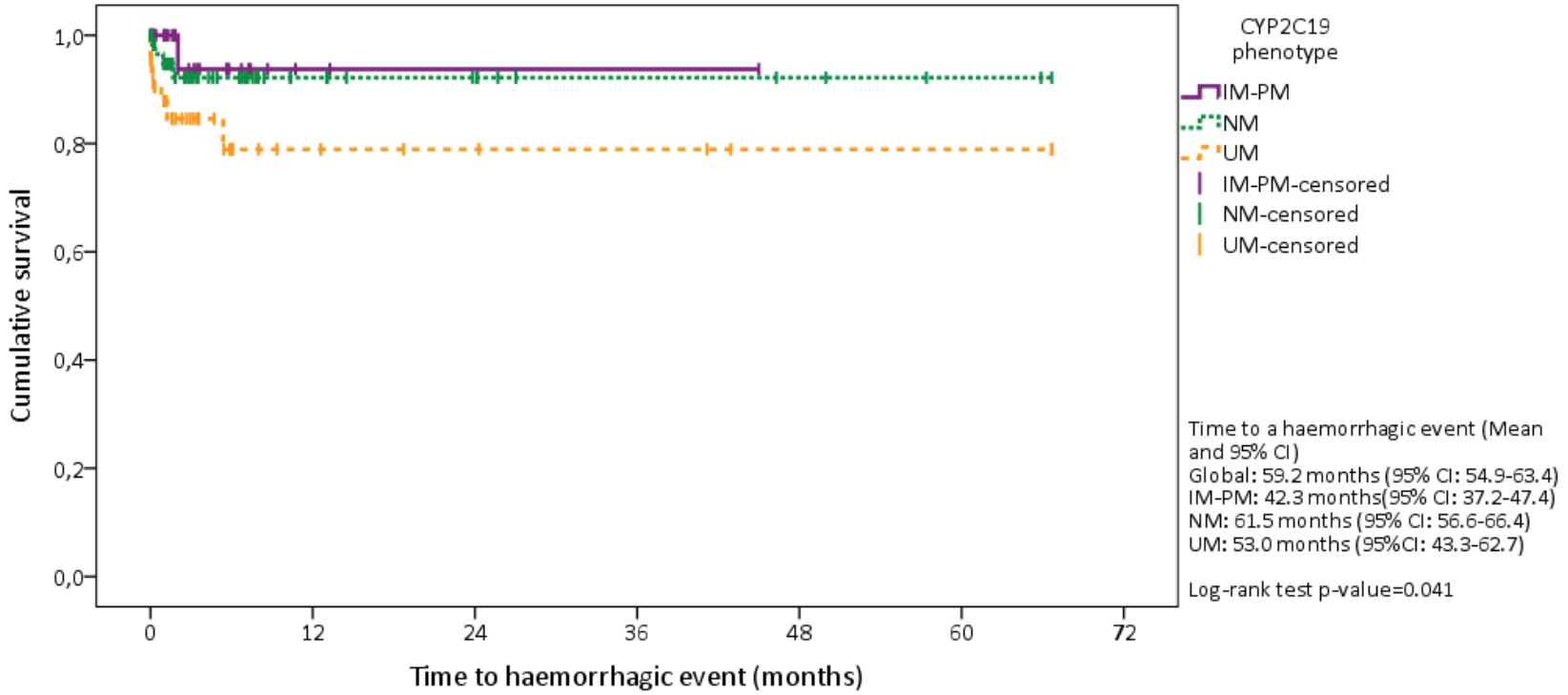
Variables predicting the incidence of ischemic events					
Ischemia Predictors	Odds-Ratio	Lower 95%CI	Upper 95%CI	p-value	Variable
CYP2C19 UM	0.060	0.003	1.076	0.056	Protective factor
Treatment duration (months)	1.041	1.007	1.077	0.017	Risk predictor
R-squared= 0.255					

Variables predicting the incidence of hemorrhagic events					
Hemorrhage Predictors	Odds-Ratio	Lower 95%CI	Upper 95%CI	p-value	Variable
CYP2C19 UM	3.60	1.071	12.1	0,038	Risk predictor
R-squared= 0.070					



	Number of patients at risk	Baseline	1 month	2 months	3 months	6 months	12 months	24 months	48 months	60 months
Phenotype	IM-PM	37	23	16	13	7	2	1	0	0
	NM	63	49	31	27	21	10	5	2	1
	UM	44	36	25	22	13	9	7	1	1

Figure 1a. The time until the appearance of an (a) ischemic or (b) hemorrhagic event studied by survival analysis with the Kaplan-Meier procedure.



	Number of patients at risk	Baseline	1 month	2 months	3 months	6 months	12 months	24 months	48 months	60 months
Phenotype	IM-PM	37	24	16	13	7	2	1	0	0
	NM	63	52	34	30	24	13	9	4	2
	UM	44	33	23	20	11	7	4	1	1

Figure 1b. The time until the appearance of an (a) ischemic or (b) hemorrhagic event studied by survival analysis with the Kaplan-Meier procedure.

Supplementary table 1.

Gene	Variant	rs number	Reference allele	Alternative allele	MAF*	Consequence	Genotyping method
ABCB1	C3435T	rs1045642	G	A	0.4	Synonymous variant	MassArray
	G2677T/A	rs2032582	C	A	0.4	Missense variant	MassArray
	G2677T/A	rs2032582	C	T	0.05	Missense variant	MassArray
	C1236T	rs1128503	G	A	0.4	Synonymous variant	MassArray
CYP1A2	*1C	rs2069514	G	A	0.21	Upstream gene variant	TaqMan
	*1F	rs762551	A	C	0.37	Intron variant	MassArray
	*1B	rs2470890	T	C	(T) 0.24	Synonymous variant	MassArray
CYP2B6	*9	rs3745274	G	T	0.32	Missense variant	TaqMan
	*5	rs3211371	C	T	0.05	Missense variant	MassArray
CYP2C8	*2	rs11572103	T	A	0.05	Missense variant	MassArray
	*3	rs10509681	T	C	0.05	Missense variant	MassArray
	*4	rs1058930	G	C	0.02	Missense variant	MassArray
CYP2C9	*2	rs1799853	C	T	0.05	Missense variant	MassArray
	*3	rs1057910	A	C	0.05	Missense variant	MassArray
CYP2C19	*2	rs4244285	G	A	0.22	Synonymous variant	LightMix
	*3	rs4986893	G	A	0.01	Stop gained	LightMix
	*17	rs12248560	C	T	0.15	Intron variant	LightSNiP
CYP3A4	*20	rs67666821	__	T	<0.001	Frameshift variant	MassArray
	*22	rs35599367	G	A	0.09	Intron variant	MassArray
CYP3A5	*3	rs776746	T	C	(T) 0.38	Splice acceptor variant	MassArray
	*6	rs10264272	C	T	0.04	Synonymous variant	MassArray
PON1	Q192R	rs662	T	C	0.46	Missense variant	MassArray
	L55M	rs854560	A	T	0.18	Missense variant	MassArray
		rs705379	G	A	0.35	Upstream gene variant	TaqMan
CES1		rs71647871	C	T	0.04	Missense variant	MassArray
P2RY12		rs2046934	G	A	(G) 0.13	Intron variant	MassArray
		rs6798347	G	A	0.29	Intron variant	MassArray
		rs6809699	C	A	0.09	Synonymous variant	MassArray
		rs9859552	G	T	0.06	Intron variant	MassArray
		rs16846673	T	C	0.02	Missense variant	MassArray
		rs6785930	G	A	0.24	Missense variant	MassArray
		rs10935838	G	A	0.13	Intron variant	MassArray
		rs5853517	__	T	0.13	Intron variant	MassArray
		rs6801273	C	T	(C) 0.42	Intron variant	MassArray
		rs6787801	G	A	(G) 0.47	Intron variant	MassArray

*Minor Allele Frequence (MAF) corresponds to the alternative allele, otherwise it is indicated in parentheses.

Supplementary table 2. Genotypic frequencies of the studied genes.

Gene	Genotype	N (%)	Gene	Genotype	N (%)	Gene	Genotype	N (%)
CYP2C19	*1/*1	63 (43.8)	CYP3A4	*1/*1	123 (87.9)	P2RY12	rs6798347	
	*1/*2	28 (19.4)		*1/*22	16 (11.4)		G/G	88 (62.9)
	*2/*2	2 (1.4)		*22/*22	1 (0.7)		G/A	46 (32.9)
	*1/*17	37 (25.7)	PON1	rs662			A/A	6 (4.3)
	*17/*17	7 (4.9)		T/T	65 (46.4)		rs6809699	
	*2/*17	7 (4.9)		T/C	56 (40.0)		C/C	105 (75.0)
CYP1A2	*1/*1B	6 (4.2)		C/C	19 (13.6)		C/A	31 (22.1)
	*1/*1F	11 (7.8)		rs854560			A/A	4 (2.9)
	*1C/*1F	49 (35.0)		A/A	64 (45.7)		rs9859552	
	*1C/*1B	1 (0.7)		A/T	54 (38.6)		G/G	93 (66.4)
	*1F/*1B	2 (1.4)		T/T	22 (15.7)		G/T	40 (28.6)
	*1B/*1B	2 (1.4)		rs705379			T/T	7 (5.0)
	*1C/*1C	51 (36.4)		G/G	31 (22.1)		rs16846673	
	*1F/*1F	18 (12.8)		G/A	69 (49.3)		T/T	140 (100)
CYP2B6	*1/*1	62 (44.3)		A/A	40 (28.6)		rs6785930	
	*1/*5	16 (11.4)	CES1	rs71647871			G/G	70 (50.0)
	*1/*9	45 (32.1)		C/C	138 (98.6)		G/A	61 (43.6)
	*5/*5	3 (2.1)		C/T	2 (1.4)		A/A	9 (6.4)
	*5/*9	3 (2.1)	ABCB1	C3435T			rs10935838	
	*9/*9	11 (7.8)		C/C	41 (29.3)		G/G	103 (73.6)
CYP2C9	*1/*1	71 (50.7)		C/T	80 (57.1)		G/A	33 (23.6)
	*1/*2	39 (27.8)		T/T	19 (13.6)		A/A	4 (2.9)
	*1/*3	21 (15.0)		C1236T			rs5853517	
	*2/*2	4 (2.9)		C/C	47 (33.6)		-/-	104 (74.3)
	*2/*3	4 (2.9)		C/T	75 (53.6)		-/T	32 (22.9)
	*3/*3	1 (0.7)		T/T	18 (12.9)		T/T	4 (2.9)
CYP2C8	*1/*1	76 (54.3)		G2677TA			rs6801273	
	*1/*2	2 (1.4)		C/C	43 (30.7)		C/C	60 (42.9)
	*1/*3	41 (29.3)		C/A	74 (52.8)		C/T	66 (47.1)
	*1/*4	13 (9.3)		C/T	8 (5.7)		T/T	14 (10.0)
	*3/*3	3 (2.1)		A/A	14 (10.0)		rs6787801	
	*3/*4	5 (3.6)		A/T	1 (0.7)		G/G	39 (27.9)
CYP3A5	*1/*3	16 (11.4)	P2RY12	rs2046934			G/A	62 (44.3)
	*1/*6	1 (0.7)		G/G	104 (74.3)		A/A	39 (27.9)
	*3/*3	121 (86.4)		G/A	32 (22.9)			
	*3/*6	2 (1.4)		A/A	4 (2.9)			

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