Influence of CYP450 Enzymes, *CES1*, *PON1*, *ABCB1*, and *P2RY12* Polymorphisms on Clopidogrel Response in Patients Subjected to a Percutaneous Neurointervention

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ABSTRACT

Purpose: Clopidogrel is a thienopyridine prodrug that inhibits platelet aggregation. It is prescribed to prevent atherothrombotic and thromboembolic events in patients receiving a stent implant in carotid, vertebral, or cranial arteries. The influence of cytochrome P-450 (CYP) 2C19 on the response to clopidogrel has been widely studied; however, the effect of other genes involved in clopidogrel absorption and metabolism has not been established in this cohort of patients.

Methods: This observational retrospective study assessed the antiplatelet response and the prevalence of hemorrhagic or ischemic events after percutaneous neurointervention in clopidogrel-treated patients, related to 35 polymorphisms in the genes encoding the clopidogrel-metabolizing enzymes (*CYP2C19*, *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP2C9*, *CYP3A4*, *CYP3A5*, carboxylesterase-1 [*CES1*], and paraoxonase-1 [*PON1*]), P-glycoprotein transporter (*ABCB1*), and platelet receptor $P2Y_{12}$. Polymorphisms were analyzed by quantitative real-time polymerase chain reaction and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry. Antiplatelet response was documented with the VerifyNow system (Accriva, San Diego, California).

Findings: We confirmed that CYP2C19 is the most important enzyme involved in clopidogrel response. The carriage of the *CYP2C19*2* allele was strongly associated with hyporesponse to clopidogrel, while the *CYP2C19*17* allele was a protective factor for the development of ischemic events (odds ratio = 0.149; $P = 0.002$) but a risk factor for bleeding (odds ratio = 3.60; $P = 0.038$). Patients carrying *ABCB1* mutated alleles showed lower aggregation values, suggesting that clopidogrel absorption is influenced by P-glycoprotein. In fact, the percentage of responders was significantly higher in the group carrying the mutated haplotype compared to the wild type (80.8% vs 43.3%; $P = 0.009$). Patients with the *CES1* G143E C/T

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genotype showed a considerably lower, aggregation value versus wild-type patients, although the difference was not significant likely due to the small sample size (59.0 [21.2] vs 165.2 [86.0] PRU; $P = 0.084$), which suggests an increased active metabolite formation. No relationship was found between polymorphisms in other *CYP* genes, *PON1*, or *P2RY12* and response to clopidogrel in patients subjected to neurointervention procedures.

Implications: Therapeutic guidelines recommend that CYP2C19 intermediate and poor metabolizers with acute coronary syndromes undergoing percutaneous coronary intervention receive an alternative antiplatelet therapy; however, genotype-guided therapy is not a standard recommendation for neurovascular conditions. This is the first study to carry out a joint analysis of *CYP2C19* and other genes involved in clopidogrel treatment in patients receiving percutaneous neurointervention. Our findings support routine genotyping in clopidogrel-treated patients. Moreover, we encourage considering an alternative antiplatelet therapy in CYP2C19 intermediate, poor and ultrarapid metabolizers. Additionally, *ABCB1* polymorphisms could be considered for a better pharmacogenetic approach. (*Clin Ther*. 2019;41:1199–1212)

Key words: *CES1*, clopidogrel, CYP2C19, hemorrhage, ischemia, *P2Y12*, *PON1*.

INTRODUCTION

Clopidogrel is one of the most widely 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] an ATP-dependent transporter encoded by *ABCB1* and located in the intestinal epithelial cell wall, which expels the drug into the intestinal lumen. Afterward, clopidogrel is extensively metabolized in the liver. Most of the parent drug (~85%) is metabolized to its inactive carboxylic acid form by carboxylesterase (CES)-1.[3] The remaining (15%) undergoes 2 sequential oxidative stages, through several cytochrome P-450 (CYP) enzymes, originating the active metabolite. First, the CYP2C19, CYP2B6 and CYP1A2 isoforms convert clopidogrel to 2-oxo-clopidogrel. Second, CYP3A4, CYP3A5, CYP2B6, CYP2C9, and CYP2C19 isoforms and the paraoxonase (PON)-1 enzyme transform 2-oxo-clopidogrel into its active form.[4,5] In addition, 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 that 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 and poor metabolizer phenotypes (IM and PM) have been associated with hyporesponsiveness to clopidogrel, since they show lower levels of active metabolite. Therefore, carriers of these variants have a higher risk for recurrent vascular events.[8–11] On the other hand, carriers of the CYP2C19 ultrarapid metabolizer phenotype (UM) have shown greater platelet inhibition and hyperresponsiveness to clopidogrel,[12–15] and consequently, an increased risk for hemorrhagic complications.[10,16,17] In this respect, the Clinical Pharmacogenetics Implementation Consortium made a series of therapeutic recommendations based on the *CYP2C19* genotype for clopidogrel-based therapy for acute coronary syndromes.[18] It is important to emphasize that the literature on clopidogrel variability in cardiologic disorders is relatively rich. However, cerebrovascular disorders addressed with neurointerventional procedures in clopidogrel treatment have not been well investigated.[19] Extrapolation is not an appropriate approach taking into account the biological background, medical condition, different therapy, risk factors, and the fact that anatomic and procedural specificities are different across diseases.[19]

Regarding neurovascular conditions, there is no genotype-guided therapy recommendation. Our group previously described an association between the CYP2C19 IM-PM phenotype and hyporesponse to clopidogrel, along with a significantly higher rate of hemorrhagic events in UM patients undergoing a percutaneous neurointervention.[20] In the current study, our aim was to evaluate the effects of the *ABCB1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5, PON1, CES1,* and *P2RY12* genes involved in clopidogrel transport,

metabolism, and mechanism of action in this cohort of neurointervened patients, including 21 new cases. This knowledge is of great importance to ensure the correct antiaggregation in these patients and to avoid the risk for subsequent events. Moreover, this research might contribute to the development of recommendation guidelines on genotype-based therapy for neurovascular conditions.

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 from Hospital Universitario de La Princesa (Madrid, Spain). The primary tolerability end point was the prevalence of either thrombotic or hemorrhagic events during treatment with clopidogrel, which could vary from a few days to several months. Other end points included: antiplatelet response, requirement of dose reduction, change in antiplatelet therapy, and treatment duration. All data were collected from the medical records and included demographic factors (age and sex), cardiovascular risk factors (eg, smoking status, hypertension, dyslipidemia, obesity, diabetes mellitus, chronic obstructive pulmonary disease, and a history of atrial fibrillation, acute myocardial infarction, and/or ischemic and hemorrhagic events), and type of intervention. Concurrent treatment with CYP2C19 inhibitors was also taken into account.

The study protocol was in compliance with the principles of the Declaration of Helsinki and with the Spain's current legislation on clinical research in humans, and was approved by the Ethics Committee of Drug Research of Hospital Universitario de La Princesa.

Assessment of Antiplatelet Response

Antiplatelet response was documented with the VerifyNow system (Accriva Diagnostics, San Diego, CA), which determined the level of platelet $P2Y_{12}$ receptor blockade (in P2Y12 reaction units; PRU) by measuring the ADP-induced aggregation (extent of platelet aggregation in the presence of $P2Y_{12}$ inhibitors). Platelet reactivity tests were performed prior to the intervention. Values below 180 PRU suggest evidence of a P2Y12 inhibitor effect, while values over 180 PRU suggest no drug effect due to low P2Y12-inhibition response. Based on these values we classified patients as responders to clopidogrel (values below 180 PRU) and nonresponders (values over 180 PRU), according to the VerifyNow reference guide. Clopidogrel dose was adjusted according to hyper- or hypotreatment response.

Genotyping

DNA was extracted from 1 mL of peripheral blood samples using a 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 35 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 shown in Supplemental Table I (see the online version at https://doi.org/10.1016/j.clinthera.2019.04.037).

CYP2C19 *2, *3, and *17 genotyping was performed by real-time quantitative polymerase chain reaction with hybridization probes (TIB MOLBIOL, Berlin, Germany) in a LightCycler 2.0 device (Roche Bioscience, Mannheim, Germany), as previously described.[20] 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 quantitative real-time polymerase chain reaction using a StepOne PCR instrument (Applied Biosystems, Foster City, California) and TaqMan assays following the manufacturer's recommendations (Applied Biosystems). The genotyping of *ABCB1, CYP1A2* (*1F and *1B*), CYP2B6*5, CYP2C8, CYP2C9, CYP3A4, CYP3A5, PON1, CES1,* and *P2RY12* was performed by matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry, with the MassARRAY platform (Agena Bioscience Inc, San Diego, California). All assays were performed with an internal quality control, with a genotyping success rate and reproducibility of 100%.

Statistical Analysis

In order to simplify the analysis, genotypes were classified according to Clinical Pharmacogenetics

Implementation Consortium's definitions of alleles[21] and allele-functionality tables, as the one described by our group for *CYP1A2*.[22] Thus, *CYP2C19* genotypes were classified according to the number of functional alleles into IM-PM (*1/*2, *2/*2, and *2/*17), normal metabolizer (NM) (*1/*1), and UM (*1/*17 and *17/*17). Moreover, *CYP2B6*, *CYP2C8*, and *CYP2C9* genotypes were also classified as PM (carriers of 2 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 the *1/*1 genotype) according to the functionality of the alleles. *CYP1A2* *1C, *1, *1B, and *1F were assigned activity scores of 0.5, 1, 1.25, and 1.5, respectively. Thus, as previously validated,[22] patients with *CYP1A2* *1/*1B, *1/*1F, *1B/*1B, *1C/*1F, and *1C/*1B genotypes were categorized as having a *CYP1A2* NM/RM (rapid metabolizer) 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 *CYP3A5* *3/*3 and *3/*6 genotypes, as *nonexpressers*. Regarding PON1, as there is no functionality table that allows for inferring a phenotype, we assigned the NM phenotype to those patients without any mutation. On the contrary, carriers of 1 mutated allele in any of the 3 variants analyzed (rs662, rs854560, and rs705379) were considered IM. Carriers of 2 mutated alleles in any of the 3 *PON1* variants were considered PM. Finally, *ABCB1* C3435T, C1236T and G2677T/A genotypes were categorized as haplotype *wild type* when there was an absence of mutation, as *heterozygote* when there was any heterozygous genotype in at least 1 of the 3 variants, and as *mutated* when there were 2 mutated alleles in any of the 3 polymorphisms.

For the analysis of the influence of all covariates, the aggregation value was translated into the variable *responders*, which classified an aggregation value of ≥180 PRU as *nonresponse* and values of <180 PRU as *response*.

Statistical analysis was performed using SPSS software version 22.0 (SPSS Inc, Chicago, Illinois); we considered P values of <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 χ^2 test. Differences in quantitative parameters between individuals were statistically analyzed by parametric univariate analysis (t test or ANOVA) or nonparametric univariate analysis (Kruskal–Wallis). A multiple regression analysis was performed to analyze the effects on aggregation value and prevalence of subsequent ischemic and hemorrhagic events. For this purpose, categorical variables with >2 categories, such as phenotype, were analyzed using dummy variables. Time to the appearance of an ischemic or hemorrhagic event was studied by means of survival analysis with the Kaplan–Meier procedure. 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 follow-up was finished.

As this study was of an observational exploratory design, we did not adjust P values for multiple comparisons, which some authors recommend.[23–25]

RESULTS

Patient Characteristics

Our study population comprised 144 patients (74 men, 70 women). Table I shows the main demographic characteristics of the patients. A total of 46.5% of the patients underwent intervention due to the presence of an aneurysm, and 53.5% presented a stenosis. While aneurysms were more frequently found in women, stenosis was more frequently detected in men.

Genotype frequencies are shown in Supplemental Table II (see the online version at https://doi.org/10.1016/j.clinthera.2019.04.037). The difference in the distribution of genotype frequencies between sexes was not significant, except for *CYP1A2*1C*, in which all the carriers were women ($P = 0.025$), and *CYP1A2*1B*, in which more women than men carried the *1/*1 genotype (32.4% vs 13.9%; $P = 0.034$).

Patient Outcome

The mean (SD) aggregation value (measured in 141 patients) was 161.3 (87.3) PRU. Men showed a higher aggregation value than that in women, although this difference was not significant (173.8 [86.0] vs 148.1 [87.3] PRU; $P = 0.081$) (Table I). According to this

Table I. Main demographic and clinical characteristics of the study population. Data are numbers (%) of patients unless otherwise noted.

Characteristic	All Patients (N = 144)	Men (n = 74)	Women (n = 70)	*P*
Age, mean (SD), years	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
Currently smoking	47 (32.6)	28 (37.8)	19 (27.1)	0.214
Diabetes mellitus	28 (19.4)	19 (25.7)	9 (12.9)	0.060
Atrial fibrillation	3 (2.1)	3 (4.1)	0	0.245
Obesity	3 (2.1)	1 (1.4)	2 (2.9)	0.612
Previous ischemic and hemorrhagic events				
Ischemic stroke	20 (13.9)	14 (18.9)	6 (8.6)	0.093
Transient ischemic attack	16 (11.1)	15 (20.3)	1 (1.4)	<0.001
Hemorrhagic stroke	11 (7.6)	1 (1.4)	10 (14.3)	0.004
Acute myocardial infarction	9 (6.3)	8 (10.8)	1 (1.4)	0.034
Reason for intervention				
Stenosis	77 (53.5)	60 (81.1)	17 (24.3)	<0.001
Aneurysm	67 (46.5)	14 (18.9)	53 (75.7)	<0.001
Type of intervention				<0.001
Stent	96 (66.7)	63 (85.1)	33 (47.1)	
Flow diverter	33 (22.9)	7 (9.5)	26 (37.1)	
Coil	12 (8.3)	2 (2.7)	10 (14.3)	
No intervention	3 (2.1)	2 (2.7)	1 (1.4)	
Concurrent treatment	141 (97.9)	73 (98.6)	68 (97.1)	0.612
ASA	139 (96.5)	73 (98.6)	66 (94.3)	0.200
PPI	110 (76.4)	56 (75.7)	54 (77.1)	0.847
SSRI	11 (7.6)	2 (2.7)	9 (12.9)	0.028
NSAID	7 (4.9)	2 (2.7)	5 (7.1)	0.266
OAC	4 (2.8)	4 (5.4)	0	0.120
Heparin	1 (0.7)	1 (1.4)	0	1.000
Patient outcome				
Aggregation value, mean (SD), PRU (n = 141)	161.2 (87.3)	173.8 (86.0)	148.1 (87.3)	0.081
Responders (n = 141)*	79 (56.0)	35 (48.6)	44 (63.8)	0.090
Change of treatment	20 (13.9)	7 (9.5)	13 (18.6)	0.149
Subsequent ischemic event	15 (10.4)	8 (10.8)	7 (10.0)	1.000
Subsequent hemorrhagic event	13 (9,0)	7 (9.5)	6 (8.6)	1.000
Dose reduction required	7 (4.9)	1 (1.4)	6 (8.6)	0.058

ASA = acetylsalicylic acid; OAC = oral anticoagulant; PPI = proton-pump inhibitor; PRU = P2Y12 reaction unit; SSRI = selective serotonin reuptake inhibitor.

* Patients with aggregation <180 PRU were considered responders.

parameter, 56% of patients were categorized as responders, with the percentage being higher in women than in men (64% vs 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 in treatment. The median treatment duration was 80 days, with a range of 1 to 3079 days.

Regarding the primary outcome, 18.8% of the patients experienced a clinical event. The prevalence of ischemic events was higher than the prevalence of hemorrhagic events (10.4% and 9%), with no significantly different distribution among sexes. The influences of aggregation values on the prevalences of ischemic and hemorrhagic events are shown in Table II. Those patients who experienced an ischemic event showed a higher aggregation value, and thus a lower response to clopidogrel, although the difference was not statistically significant. The difference in aggregation values between patients with or without a hemorrhagic event was not significant. Moreover, of those patients considered clopidogrel responders (PRU <180), 7.6% experienced an ischemic event during clopidogrel treatment, while this percentage was higher in clopidogrel nonresponders (PRU ≥180; 14.5%), although this difference was not significant ($P = 0.271$). Regarding hemorrhagic events, the prevalences were similar between responders and nonresponders (7.6% vs 6.5%; $P = 1.000$)."

During the neurointerventional procedures, 4 patients experienced a hemorrhage due to an artery perforation. Two of them were CYP2C19 IM-PM, one was CYP2C19 NM and 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 III shows a summary of the patients' outcome according to the different genotypes/phenotypes of the analyzed genes.

Table II. Influence of the aggregation value on the prevalence of ischemic and hemorrhagic events.

Event Type	Aggregation Value, Mean (SD), PRU	*P*
Ischemic event		0.244
Yes (n = 15)	186.1 (90.2)	
No (n = 126)	158.3 (86.8)	
Hemorrhagic event		0.699
Yes (n = 10)	150.9 (89.1)	
No (n = 131)	162.0 (87.4)	

PRU = P2Y12 reaction unit.

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 significantly worse response to clopidogrel (Table III). This lack of response may explain the significantly shorter treatment duration in these patients (nonstandardized β coefficient = –235.6; $P = 0.027$).

Moreover, regarding the primary outcome, the prevalence of ischemic events was lower in the UM group compared to IM-PM and NM (2.3% vs 10.8% and 15.9%, $P = 0.060$). Figure 1A shows the time to the appearance of an ischemic event, with a significant difference between the survival functions of the 3 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 significant difference in UM compared to NM ($P = 0.017$). Moreover, Haberman-corrected typed residues pointed to a significantly lower frequency of ischemic events in the UM group (2.3%) than that 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 prevalence of hemorrhagic events was detected in the UM group (15.9%), followed by NM (7.9%) and IM-PM (2.7%), although these differences did not reach statistical significance ($P = 0.101$). The hemorrhagic event onset times are presented in Figure 1B, showing a difference between the survival functions of the 3 phenotypes ($P = 0.041$). However, the comparison by pairs with statistical log-rank did not detect significant differences (IM-PM vs NM, $P = 0.547$; IM-PM vs UM, $P = 0.078$; NM vs UM, $P = 0.097$). Nonetheless, 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.

Table III. Outcomes of patients undergoing percutaneous neurointervention according to the studied genes.

Gene	Genotype/ Phenotype/ Haplotype	Aggregation Value, Mean (SD), PRU (n = 141)*	Responders,† No. (%)	Ischemic Events, No. (%)	Hemorrhagic Events, No. (%)
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)	5 (7.9)
	UM (n = 44)	157.9 (76.8)	24 (57.1)	1 (2.3)	7 (15.9)
	P	0.004	0.007	0.06	0.109
CYP2C9	PM-IM (n = 69)	153.7 (85.9)	39 (56.5)	11 (15.9)	5 (7.2)
	NM (n = 71)	173.8 (86.3)	36 (52.9)	4 (5.6)	8 (11.3)
	P	0.173	0.733	0.059	0.563
CYP2C8	PM-IM (n = 64)	150.7 (85.7)	38 (59.4)	10 (15.6)	6 (9.4)
	NM (n = 76)	175.1 (86.0)	37 (50.7)	5 (6.6)	7 (9.2)
	P	0.100	0.390	0.104	1.000
CYP1A2	PM (n = 1)	60	1 (100)	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)	6 (8.7)
	P	0.303	0.201	0.814	1.000
CYP2B6	PM (n = 17)	170.1 (65.3)	11 (64.7)	3 (17.6)	2 (11.8)
	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	0.474	0.518	0.508	0.646
CYP3A4	PM-IM (n = 17)	148.7 (99.1)	11 (68.8)	3 (17.6)	3 (17.6)
	NM (n = 123)	165.7 (84.7)	64 (52.9)	12 (9.8)	10 (8.1)
	P	0.464	0.29	0.395	0.196
CYP3A5	Nonexpressers (n = 123)	161.4 (88.1)	68 (56.7)	12 (9.8)	12 (9.8)
	Expressers (n = 17)	179.6 (73.6)	7 (41.2)	3 (17.6)	1 (5.9)
	P	0.418	0.3	0.395	1
CES1 rs71647871	C/C (n = 138)	165.2 (86.0)	71 (54.1)	15 (10.9)	13 (9.4)
	C/T (n = 2)	59.0 (21.2)	2 (100)	0	0
	P	0.084	0.501	1	1
PON1	PM (n = 114)	165.3 (86.6)	59 (52.7)	15 (13.2)	11 (9.6)
	IM (n = 19)	164.1 (86.3)	11 (57.9)	0	1 (5.3)
	NM (n = 7)	130.2 (90.6)	5 (83.3)	0	1 (14.3)
	P	0.626	0.358	0.184	0.700
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)	9 (10.8)
	Mutated (n = 27)	151.7 (80.9)	21 (80.8)	1 (3.7)	2 (7.4)
	P	0.727	0.009	0.478	0.922
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
	P	0.825	1	1	1

IM = intermediate metabolizer, NM = normal metabolizer, PM = poor metabolizer, RM = rapid metabolizer, UM = ultrarrapid metabolizer.

* Aggregation values were available in 141 patients with *CYP2C19* genotype and in 137 patients evaluated for the rest of the genes.

† Patients with aggregation <180 P2Y12 reaction units were considered responders.

Influence of Other CYP Enzymes, *CES1* and *PON1* on Patient Outcome

There was no influence of *CYP2C9, CYP2C8, CYP1A2, CYP2B6, CYP3A4, CYP3A5,* or *PON1* on aggregation values (Table III).

Concerning *CES1*, although it did not reach statistical significance, we observed that patients carrying the G143E (rs71647871) C/T genotype showed considerably lower aggregation compared to wild-type patients (59.0 [21.2] vs 165.2 [86.0] PRU; $P = 0.084$).

Regarding the primary outcome, the prevalence of ischemic events was higher with the *CYP2C9* PM-IM phenotype (15.9%) compared to that with NM (5.6%), but the difference was not statistically significant ($P = 0.059$). Nonetheless, no other CYP enzyme, nor *CES1* or *PON1*, had a significant influence on the prevalence of ischemic or hemorrhagic subsequent events.

Influence of *ABCB1* on Patient Outcome

There was a tendency toward lower aggregation in patients carrying *ABCB1* C3435T, C1236T and G2677T/A mutated alleles. In fact, the percentage of responders was significantly higher in patients carrying the mutated haplotype ($P = 0.009$) (Table III). However, there was no association between *ABCB1* haplotypes and the prevalence of ischemic or hemorrhagic events.

Influence of *P2RY12* on Patient Outcome

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

Influence of the Concurrent Treatment With Proton-Pump Inhibitors on Patient Outcome

In all, 76.4% of the patients were receiving proton-pump inhibitors (PPIs), which are CYP2C19 inhibitors, as concurrent treatment. Of these, 50% were receiving omeprazole and 50% pantoprazole. Patients on PPI treatment showed a significantly higher aggregation value compared to those without PPI treatment (170.7 [84.5] vs 129.0 [90.2] PRU; $P = 0.017$). Moreover, both patients receiving omeprazole and pantoprazole showed similar aggregation values (170.8 [84.1] vs 170.6 [85.8] PRU, respectively). However, the concurrent treatment with PPIs had no influence on the prevalence of either ischemic events (10.9% of patients receiving PPIs vs 8.8% of patients not receiving PPIs; $P = 0.768$) or hemorrhagic events (9.1% vs 5.9%; $P = 0.732$).

Results From the Multivariate Analysis

A multiple regression analysis was performed considering the aggregation value, the response rate,

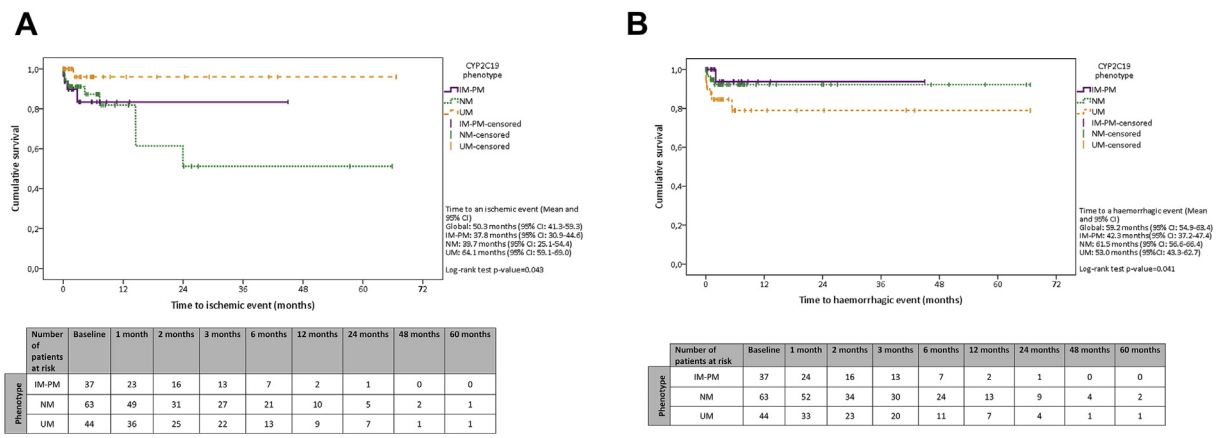

Figure 1. Times to the appearance of ischemic (A) or hemorrhagic (B) events studied by Kaplan–Meier survival analysis.

and the prevalence of ischemic and hemorrhagic events as dependent variables. It included sex, age, polymorphisms in all of the genes analyzed, presence of cardiovascular risk factors (hypertension, dyslipidemia, obesity, atrial fibrillation, diabetes mellitus, and smoking status), previous ischemic or hemorrhagic events, type of intervention, and concurrent treatment with PPIs as independent variables. A summary of the results is shown in Tables IV and V.

Briefly, age, PPI concurrent treatment, and *CYP2C19* IM-PM phenotype appeared to be predictors of a worse response due to a higher aggregation value. In addition, receiving intervention with a flow diverter was a predictor of a better response compared to stent and coil interventions. These 4 factors explained 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 remained as predictors of worse response. Additionally, *ABCB1* mutated haplotype appeared as a predictor of a better response ($r^2 = 0.433$).

In addition, as shown in Table V, *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% to 30% of patients are nonresponders.[26] Therefore, these patients are at increased risk for ischemic events after stent implantation.[27] Factors such as age, body mass index, comorbidities, concurrent treatment, and compliance explain <10% of this variability.[28] Consequently, the role of genetics 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 observed in our previous work.[29] Our results contradict those of Lin et al,[30] who found that the carriage of the *CYP2C19*17* allele was associated with the prevalence of ischemic events. In fact, we previously showed an increased risk for bleeding in *CYP2C19* UM patients.[29,31] which Haberman-corrected typed residues confirmed in the current study with a larger sample size. We encourage considering this fact for the treatment approach in these patients.

Furthermore, Zhu et al[32] established a correlation between *CYP2C19* no-function alleles and an increased risk for subsequent ischemic events in patients subjected to stent implantation in the carotid artery. However, in our study, we could not confirm this fact, probably due to the small sample size. Moreover, carriers of the *CYP2C19*2* allele are more closely evaluated in clinical practice. In fact, with the vast amount of evidence which confirms a higher risk of ischemic events in those patients, most clinicians decide to change clopidogrel for an alternative therapy to avoid the risk for subsequent events. Conversely, what we could confirm is the

Table IV. Multivariate analysis: variables influencing aggregation value.

Aggregation Value Predictor	Nonstandardized β Coefficient	*P*	Semipartial 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
PPI treatment	38.404	0.029	0.181	3.2	26.4	Worse response

IM-PM = intermediate metabolizer—poor metabolizer; PPI = proton-pump inhibitor.
* Adjusted $r^2 = 0.327$.

Table V. Multivariate analysis: variables influencing P2Y12 receptor blockade, ischemia, and hemorrhage.

Response Predictor	Odds Ratio	Lower 95% CI	Upper 95% CI	*P*	Variable
Variables contributing to 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 mutation	6.298	1.555	25.499	0.010	Better response
Ischemia predictors†					
CYP2C19 UM	0.060	0.003	1.076	0.056	Protective factor
Treatment duration (mo)	1.041	1.007	1.077	0.017	Risk predictor
Hemorrhage predictors‡					
CYP2C19 UM	3.60	1.071	12.1	0,038	Risk predictor

IM-PM = intermediate metabolizer—poor metabolizer; UM = ultrarapid metabolizer.
* Patients with aggregation >179 PRU were considered nonresponders. r^2= 0.433.
† r^2= 0.255.
‡ r^2 = 0.070.

premise by Colley and Yan,[1] who published a revision about the association between the carriage of the *CYP2C19*2* allele and hyporesponse to clopidogrel in neurointervened patients.[1] This circumstance was observed in our previous work with a lower sample size.[31]

In addition, Moore et al[33] analyzed the efficacy, tolerability, and cost-effectiveness of clopidogrel treatment compared to ticagrelor in patients with cerebral aneurysms treated with flow diverter. They found that ticagrelor was not inferior in preventing thromboembolic complications. However, due to the much higher cost of ticagrelor, this alternative therapy should be used only in clopidogrel nonresponders. Based on our results, we suggest that patients with the *CYP2C19* IM-PM or UM phenotype receive an alternative antiplatelet therapy.

Influence of Other CYP Enzymes, *CES1* and *PON1*

There is controversy whether there is an association of the most studied *CYP2C9* alleles (*2 and *3) and clopidogrel effect. Some authors state no significant relationship,[12] while others associate the presence of the *3 allele with a higher prevalence of stent thrombosis.[34] In our study, *CYP2C8* and *CYP2C9* were not associated with a difference in clopidogrel response. However, we observed a tendency toward a higher prevalence of ischemic events in subjects carrying *CYP2C8* or *CYP2C9* PM- IM phenotype, which was not statistically different either in univariate or in multivariate analyses. Further approaches with larger sample sizes would be of interest.

Reduced CYP3A4 activity was associated with an increased risk for stent thrombosis in patients with acute coronary syndrome treated with clopidogrel.[35] 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.[36] However, in our study, CYP3A4 and CYP3A5 showed no significant role in explaining some of the response variability, since a large portion of CYP3A variability is not accounted for by the genetic polymorphisms chosen in our study. Nonetheless, our results resemble those of Holmberg et al,[37] who found that neither *CYP3A4* nor *CYP3A5* genotype affected clopidogrel AUC or platelet inhibition in healthy volunteers.

Furthermore, the *CES1* G143E polymorphism (rs71647871) has been associated with a decreased protein functionality.[38] Lewis et al[39] 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 artery disease. Consistent with the findings from those studies, we found a tendency toward 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 find only 2 carriers of the G143E defective allele. Further research is needed in this cohort of patients to confirm whether there is an association.

Regarding *PON1*, the Q192R polymorphism (rs662) was described to condition the active metabolite formation.[5] In our study, we observed that patients carrying defective *PON1* alleles (assigned as PM and IM) showed a tendency toward a higher aggregation value, although the difference was not significant. This fact is consistent with findings from the study previously reported by Verschuren et al,[40] who found that patients carrying the rs662 defective allele may have lower levels of active metabolite, thus resulting in a poorer response and an increased risk for ischemic events. In our study, the prevalence of ischemic events was higher in the PM group, but the difference was not significant and deserves further research. However, there is controversy on whether clopidogrel is a PON1 substrate, since other authors have stated that there is no relationship between Q192R and differences in pharmacologic and clinical clopidogrel response.[41,42]

Influence of *ABCB1*

It has been previously suggested that the most studied *ABCB1* polymorphism, C3435T, has no influence on clopidogrel response or risk for stent thrombosis in patients undergoing coronary stenting.[43] However, Taubert et al[2] 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. 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 could be explained by higher concentrations of clopidogrel and its metabolite, since the efflux pump would be working inefficiently. Some studies relate *ABCB1* C3435T polymorphism to a lower P-gp expression in minor allele carriers.[44–47] Moreover, an *in vitro* study found an association between the T minor allele and altered protein folding and reduced activity.[48] 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 $P2Y_{12}$, some polymorphisms (rs10935838, rs2046934, rs5853517, and rs6809699) have been associated with enhanced platelet reactivity.[49,50] However, these associations have not been replicated and the level of evidence is low. In our study, we could not find a significant association between *P2RY12* haplotypes and clopidogrel response. The lack of association between *P2RY12* polymorphisms and clopidogrel response matches the results from Giusti et al[9] and Cuisset et al.[51] They demonstrated that *P2RY12* rs2046934 was not associated with antiplatelet activity in clopidogrel-treated patients with acute coronary syndrome. Moreover, Simon et al[52] described no association between rs16846673, rs6809699, and rs6785930 and the risk for adverse cardiovascular events in patients with acute myocardial infarction receiving clopidogrel.

Study Limitations

The main limitation of the present study was the unfeasibility of measuring clopidogrel and its active metabolite concentrations, which could have been useful to correlate it with patients' aggregation values and clinical outcomes. 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, such as *CES1* polymorphisms. Hence, further investigation is warranted. Another possible limitation was the lack of multiple-comparisons correction, which could have led to false-positive results. However, some experts recommend not correcting for multiple testing when analyzing data.[23–25] Indeed, it is recommended to account for multiple comparisons when interpreting the results, rather than in calculations. In fact, some authors state that the use of multiple-comparisons correction should be avoided in performing empirical research, since there is a potential cost of many more false-negatives when controlling for false-positives.[53,54]

CONCLUSIONS

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

CONFLICTS OF INTEREST

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APPENDIX

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	2 (1.4)		A/A	64 (45.7)		rs9859552	
	*1C/*1B	2 (1.4)		A/T	54 (38.6)		G/G	93 (66.4)
	*1F/*1B	51 (36.4)		T/T	22 (15.7)		G/T	40 (28.6)
	*1B/*1B	49 (35.0)		rs705379			T/T	7 (5.0)
	*1C/*1C	1 (0.7)		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)			