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Pharmacogenetic Impact of *VKORC1* and *CYP2C9* Allelic Variants on Warfarin Dose Requirements in a Hispanic Population Isolate

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Warfarin is the most prescribed oral anticoagulant worldwide. Because of the complexity of warfarin therapy, we attempted to dissect genetic from bioenvironmental factors influencing warfarin dose responses in individuals of a genetic isolate of Hispanic ancestry. A total of 191 patients with standard values of international normalized ratio were recruited. Three groups with a significantly different warfarin dose response were identified, that is, sensitive (2.28 $\pm$ 0.50 mg/d), intermediate (4.2 $\pm$ 0.76 mg/d), and resistant (7.40 $\pm$ 1.54 mg/d; Tukey test, $P < .001$). Age had a significant inverse correlation with warfarin dose ($P < .001$; effective dose diminished 0.56 mg/d/decade). Required doses were higher for individuals with *CYP2C9* variants containing the allele **1* compared to those individuals with variants composed of other alleles ($P = .006$). Similarly, individuals with *VKORC1*-1639GG and *VKORC1*-1639GA genotypes also required higher doses compared to the *AA* genotype ($P < .001$). Evaluation of potential gene-gene interactions between *CYP2C9* and *VKORC1* polymorphisms showed significant differences in dosing for *CYP2C9* genotypes within the *VKORC1*-1639G/A subgroup ($P = .013$). A stepwise multivariate linear regression analysis showed that 38.2% of the warfarin dose response variance was accounted for by a model involving age (20.9%), *VKORC1*-1639G/A (11.3%), and *CYP2C9*1, *2*, and **3* variants (7.1%). These results corroborate previous findings on warfarin pharmacogenetics and define a contrastable gene-bioenvironment interaction model suited to be used in Hispanic populations.

Keywords: warfarin; *CYP2C9*; *VKORC1*; Colombia; genetic isolate; pharmacogenetics

Introduction

Warfarin is the world's most prescribed oral anticoagulant for the prophylaxis and treatment of thromboembolic disease.[1] Its clinical use is very complex and it has (1) a narrow therapeutic range that makes frequent monitoring essential to avoid adverse effects;[1] (2) possible adverse effects that involve bleeding complications (0.12%/year incidence) even when used within therapeutic range;[2] (3) a wide response of interindividual variability associated with bioenvironmental factors, such as concurrent medication, age, gender, body weight, body mass

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index (BMI), body surface area (BSA), height, and race;[3-6] and (4) differential pharmacogenetics associated with polymorphisms harbored at genes such as the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 CYP2C9 (*CYP2C9*) genes.[7-12]

Warfarin S-enantiomer, the most active pharmacological form, is mainly metabolized by the cytochrome P450 CYP2C9 enzyme.[13] Single nucleotide polymorphisms (SNPs) harbored at the coding region of the *CYP2C9* gene, known as *CYP2C9*2* (rs1799853; R144C) and *CYP2C9*3* (rs1057910; I359L), have been associated with reduced catalytic activity (12% and 3%, respectively) when compared to the wild type (*CYP2C9*1*)[13-17] and are clinically associated with longer periods to achieve stable dosing and a higher risk of bleeding events. Variants of the recently cloned *VKORC1* gene are another major factor affecting warfarin requirements.[7] The VKORC1 protein is the warfarin target and is part of a complex that recycles vitamin K, an essential cofactor for the activation of the coagulation factors catalyzed by the γ-glutamyl carboxylase enzyme (GGCX).[7] The *VKORC1*-1639/G allele of SNP rs9923231, harbored at the E-box of *VKORC1* promoter region, was associated with a 44% higher catalytic activity compared to the *A* allele.[18] Therefore, higher doses of warfarin are required in patients carrying the *G* allele.[18,19] Racial differences in *VKORC1* haplogroup frequencies account for warfarin variance, a response that requires low, intermediate, and high doses in Asian, Caucasian, and African populations, respectively.[10,19,20]

This study aims to provide a genetic-bioenvironmental testable model outlining the response to anticoagulant treatment with warfarin in subjects of Hispanic ancestry. Our rationale for using this population is that the genetic homogeneity present in this very well-characterized genetic isolate will provide a better dissection of gene, environment, and interacting effects on warfarin dose requirements.

Materials and Methods

Participants

Between January 1 and July 31, 2006, we ascertained 191 unrelated subjects on warfarin treatment at the Vascular Service of the Hospital Universitario San Vicente de Paul (HUSVP), located in Medellin, Colombia, South America. Genetic, geographic, and demographic features of this self-designated, genetically isolated "Paisa" community have been described in detail elsewhere.[21-24] Today, most people in the State of Antioquia belong to the self-designated "Paisa" community. It is very important to point out that this genetic isolate is really a community dispersed from north to south in Colombia.[22] The Paisas, located between the central and western branches of the Andes Mountains, share cultural and demographic features. Their ethnohistorical origin stems most likely from Spaniards, Sephardic Jews (a subgroup of Jews originating in the Iberian Peninsula), and Basques.[24] The admixture with the African and Amerindian populations has been historically documented as low.[24,22] Several lines of genetic evidence show that this community exhibits the features of a genetic isolate. First, using the identity coefficient method, the ancestral ethnic components have been estimated as 85% Caucasian and 15% Amerindian.[22]

Information regarding age, gender, concurrent medication, diagnoses, daily doses, and international normalized ratio (INR) values were obtained from the patients' medical records. Individuals with an INR in the range of 1.8 to 3.2 for at least 1 month after 2 consecutive measurements were defined as patients.[1] Weight and height were measured during the same clinical visit when informed consent and venous blood samples were obtained for further genotyping. Body surface area was calculated using the Dubois & Dubois formula; $BSA = (W^{0.425} \times H^{0.725}) \times 0.007184$. Body mass index was calculated as W/H^2, where W is the weight in kilograms and H is the height in centimeters. Potential effects of concurrent medication (losartan, omeprazole, acetylsalicylic acid, and amiodarone) on effective warfarin doses were considered and daily doses of these medications were recorded to control for these potential confounders during statistical analyses. We only selected those medications that were systematically and continuously prescribed and administrated by the Vascular Service of the Hospital Universitario San Vicente de Paul, to accomplish with homogeneity and reliability of the data. Warfarin clinical indications were grouped according to Moridani et al (Table 1).[25] As exclusion criteria, we considered liver disease, pregnancy, and hospitalization. Liver disease was evaluated by measuring the mean levels of albumin, alanine aminotransferase, and platelet counts. The presence of any significant deviation from standard values for the reference population attended at the Hospital Universitario San Vicente de Paul, was indicative of exclusion. Hospitalization refers to any inpatient period at any time. A control group of 163 unrelated,

Table 1. Demographic and Clinical Features of Stable Patients as Defined by INR

Age, mean ± SD (95% CI)	
Stable population	54.31 ± 17.20 (51.18-57.45)
Sex	
Female N (%)/male N (%)	70 (58.82)/48 (40.34)
Daily warfarin dose, mean ± SD, mg/d (95% CI)	
Dosage of stable population	4.47 ± 2.09 (4.11-4.87)
Dosage of sensitive patients	2.28 ± 0.49 (2.10-2.46)
Dosage of intermediate patients	4.20 ± 0.76 (3.99-4.40)
Dosage of resistant patients	7.40 ± 1.54 (6.82-7.98)
INR, mean ± SD (95% CI)	2.58 ± 0.29 (2.52-2.63)
Clinical indication, mean ± SD, mg/d (N)	
Mechanical prosthesis (MP)	4.88 ± 2.15 (38)
Atrial fibrillation (AF)	3.39 ± 1.75 (18)
Deep vein and arterial thrombosis (DVT/AT)	4.44 ± 2.18 (41)
Others[a]	4.67 ± 1.87 (21)
Concurrent medication N (%)	
Losartan	2 (2.27)
Omeprazole	9 (10.23)
Amiodarone	5 (5.68)
Acetylsalicylic acid	1 (1.14)
Total stable patients analyzed	119

NOTES: CI = confidence interval; INR = international normalized ratio.

[a] Aortic lesion, coronary disease, mitral lesion, cerebral vascular disease, congestive heart failure, mitral stenosis, auricular flutter, acute cerebral infarction, chronic obstructive pulmonary disease, and antiphospholipid syndrome.

healthy young subjects were analyzed for each SNP to further examine the population's allele frequencies in order to avoid biases related to clinical conditions leading to warfarin treatment.

Genotyping

DNA was extracted by a conventional salting out method from peripheral blood samples (3-4 mL) collected in EDTA. Genotyping of *CYP2C9*2* (rs1057910), *CYP2C9*3* (rs1799853), and *VKORC1*-1639G/A (rs9923231) polymorphisms was performed using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assays.[26] Conditions for the genotyping of the *VKORC1*-1173C/T (rs9934438) SNP were as follows: 25 μL final reaction volume that contained 1× Taq polymerase buffer, 1 U Taq polymerase, 1.5 mmol/L $MgCl_2$ (New England Biolabs, MA, USA), 2 μL genomic DNA (200 ng/μL), 200 μmol/L dNTPs, 0.5 μmol/L of the reverse primer 5′-TTCCAAGAAGCCACCTGGGC-3′, and 0.5 μmol/L of the redesigned forward primer 5′-TGACATGGAATCCTGACGTG-3′. Reactions were run at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 56°C for 40 seconds and 72°C for 40 seconds, and a final amplification step of 72°C for 10 minutes. The 110-bp product was digested with 3 U of Sty I (New England Biolabs, MA, USA) at 37°C for 16 hours. The 56-bp and 54-bp fragments were separated on 2.5% agarose and visualized by ethidium bromide staining.

Statistical and Genetic Analyses

Allele frequencies were estimated by gene counting. Hardy-Weinberg Equilibrium (HWE) was tested using a χ^2 test. Case-control potential differences at genotype and allelic levels were also tested by a χ^2 test. These frequencies were compared with those reported for Spaniard populations.[27] Linkage genotype disequilibrium (LD) between *VKORC1*-1639G/A and *VKORC1*-1173C/T SNPs was tested by the Fisher test, as implemented in GenPop 3.3. *CYP2C9* genotypes were grouped as follows according to the number of alleles with impaired warfarin metabolism (dominant model): group A = *CYP2C9*1*1*, group B = *CYP2C9*1*2* and **1*3*, and group C = *CYP2C9*2*2* and **2*3*. The *CYP2C9*3*3* genotype was not found in this population. Warfarin requirements were logarithmically transformed to accomplish normality. Association between dosage and categorical variables such as gender, diagnose, and genotypes was examined by a 1-way analysis of variance (ANOVA), applying the Harley, Cochran's C, and Bartlett homogeneity, and the Newman-Keuls and Tukey tests. Correlations between warfarin dose and the continuous variables age, BSA, and BMI were assessed by simple linear regression analysis. Stepwise multivariate linear regression was performed to model the relationship between dose and each variable measured to quantify the proportion of explained variance by the coefficient of determination R^2. Epistasis between loci was tested under the null hypothesis that the effect of *CYP2C9* genotype on dose requirements was equal in all VKORC1 groups. The alternative epistasis hypothesis contrasted an interaction term (VKORC1 × CYP2C9) using the *F* test.

Table 2. Differences in Daily Warfarin Dose Requirements of Bioenvironmental and Genetic Variable

Variable		N	Mean ± SD, mg/d (95% CI)	*P* value
Categorical factors				
VKORC1-1639G/A	GG	38	5.17 ± 0.33 (4.46-5.89)	<.001
	GA	56	4.57 ± 0.28 (4.00-5.14)	
	AA	26	3.38 ± 0.40 (2.68-4.08)	
CYP2C9	**1*1*	83	4.80 ± 0.23 (4.35-5.34)	.0064
	**1*2/*1*3*	28	3.79 ± 0.40 (3.17-4.26)	
	**2*2/*2/*3*	2	1.79 ± 1.79 (1.21-3.78)	
Clinical indication	AF	18	3.39 ± 1.75 (2.52-4.26)	.0185
	MP	37	4.88 ± 2.15 (4.22-5.66)	
	DVT/AT	41	4.44 ± 2.18 (3.75-5.12)	
	Others[a]	22	4.67 ± 1.87 (3.81-5.41)	
Gender	Female	70	4.47 ± 2.06 (3.98-4.96)	.77
	Male	48	4.46 ± 2.18 (3.84-5.10)	
Continuous factors	r^2	Correlation	Regression equation	*P* value
Age	.200	−.45	$y = 7.43–0.06$ (age)	<.001
BSA	.019	.14	$y = 1.92+1.49$ (BSA)	.13
BMI	.0009	.0306	$y = 4.10+0.01$ (BMI)	.74

NOTES: AF = atrial fibrillation; BMI = body mass index; BSA = body surface area; CI = confidence interval; DVT/AT = deep-venous thrombosis and arterial thrombosis; MP = mechanical prosthesis.

[a] Aortic lesion, coronary disease, mitral lesion, cerebral vascular disease, congestive heart failure, mitral stenosis, auricular flutter, acute cerebral infarction, chronic obstructive pulmonary disease, and antiphospholipid syndrome.

We calculated the 25th and 75th percentiles of mean daily doses to identify the sensitive, intermediate, and resistant groups of patients. Dose ranges resulted from the 25th (≤2.86 mg/d) and 75th (≥5.54 mg/d) percentiles of mean daily doses of our study population. Genetic and nongenetic predictors of warfarin dose ranges were evaluated in 2 separate multivariate logistic regression analyses: one determining predictors of low-dose range and the other high-dose range. Odds ratios (ORs) and confidence intervals (CIs) were obtained from the logistic regression tests. All statistical tests were performed with SPSS 14.0. A *P* value of less than .05 was considered statistically significant.

Ethical Issues

The HUSVP Ethics Committee approved the study, and both patients and control individuals provided written informed consent for the study.

Results

Patients' demographic and clinical features are summarized in Table 1. The mean age of patients was 54.31 ± 17.20. Females accounted for 58.82% of the patients. The mean stable daily warfarin dose was 4.47 ± 2.09 mg with a mean INR of 2.58 ± 0.29 (range 1.93-3.26). Considering the warfarin indication, the most frequent diagnosis was deep-venous thrombosis and arterial thrombosis (DVT/AT; 34.75%), followed by mechanical prosthesis (MP; 32.20%) and atrial fibrillation (AF; 15.25%). Concurrent medications known to influence warfarin dosing were detected in 20% of cases but failed to show any effect on dose requirements ($P = 0.79$; Table 1).

Both the mean daily dose for sensitive, intermediate, and resistant groups (2.28 ± 0.49, 4.20 ± 0.76, and 7.40 ± 1.54 mg/d, respectively) and the average age (63.16 ± 16.13, 52.88 ± 16, and 47.10 ± 17.07 years, respectively) were significantly different (Tukey test, $P < .001$ and $P < .001$, respectively).

Simple correlation analysis of the data showed that warfarin dose was significantly and inversely correlated with age ($r = -.45$; $P < .001$), with a slope of 0.55 mg/d/decade (Table 2). However, BSA and BMI were not related to daily warfarin dose requirements ($P = .13$ and $P = .74$, respectively). Using ANOVA, we found differences among different diagnoses ($P = .02$), with AF being the clinical indication with the lowest dose requirement. Gender had no significant effect on the daily maintenance dose of warfarin with an average of 4.47 ± 2.06 mg (range 1.43-9.64 mg) for females and 4.48 ± 2.18 (1.07-11.07 mg) for males (ANOVA, $P = .77$; Table 2).

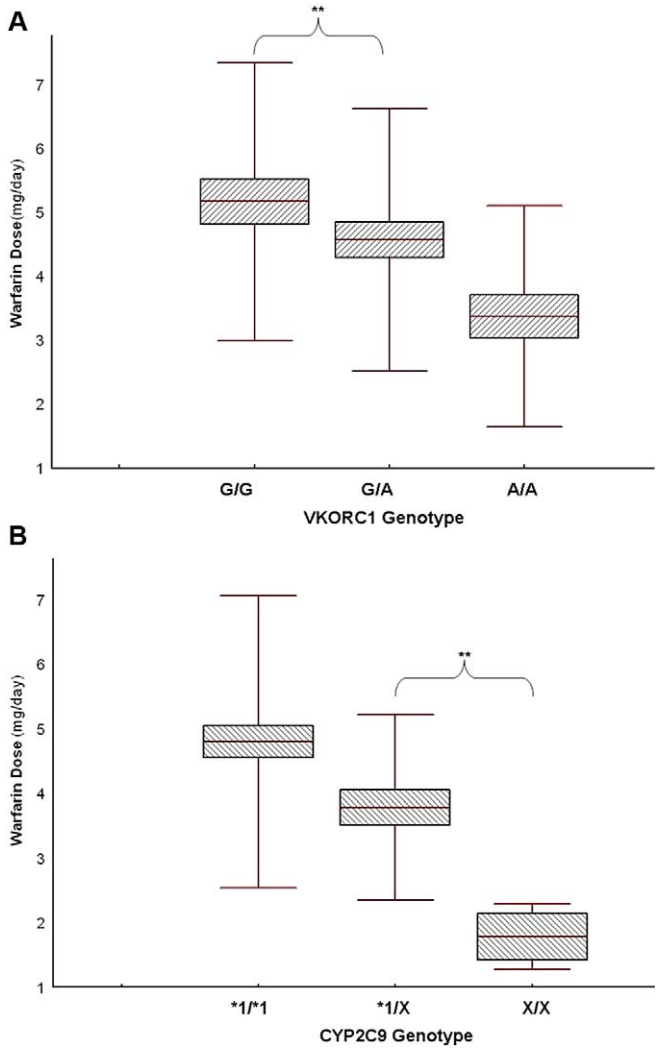

Figure 1. The influence of *VKORC1*-1639G/A and *CYP2C9* genotypes on daily warfarin maintenance dose. A, GG and GA genotypes require significantly more dose than *AA* genotype ($P < .001$); B, *CYP2C9*1*1* genotype needs more warfarin than *CYP2C9*1/x* and *CYP2C9x/x* genotypes. x = any of *CYP2C9*2* and *CYP2C9*3* alleles ($P = .006$).

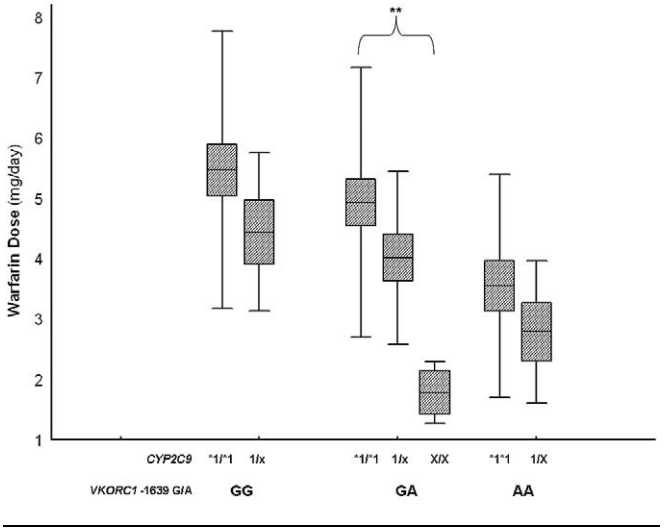

Figure 2. Interaction between *CYP2C9* and *VKORC1* polymorphisms on daily dose requirements. Boxes indicate the mean and standard error. Vertical lines above and below boxes indicate SD. Mean dose showed significant differences among *CYP2C9* genotypes within the *VKORC1*-1639GA subgroup ($P = .013$), while it was not significant within GG and AA subgroups ($P = .610$ and $P = .380$, respectively).

No significant deviation from HWE was observed for the genotyped SNPs. The VKORC1-1639G/A and VKORC1-1173C/T polymorphisms were in strong LD ($P < .001$) and consequently, statistical analyses were performed in relation to one of them. We chose VKORC1-1639G/A because of the suggested functional effects of the polymorphism on the promoter activity. This SNP had a significant effect on daily warfarin dose requirement (ANOVA, $P < .001$). Homozygous (GG) and heterozygous (GA) patients required higher doses than patients with the AA genotype (GG = 5.17 $\pm$ 0.33 mg, GA = 4.57 $\pm$ 0.28 mg, and AA = 3.38 $\pm$ 0.40 mg; Table 2 and Figure 1A). We did not find differences between cases and controls for allelic frequencies of the VKORC1-1639G/A polymorphism (Table 2).

CYP2C9 genotypes also influenced warfarin requirements. Patients with CYP2C9*1*1 genotype required more warfarin per day than heterozygous and homozygous patients for the polymorphisms with metabolic deficit (CYP2C9*2 and *3; CYP2C9*1*1 = 4.80 $\pm$ 0.23 mg, CYP2C9*1/x = 3.79 $\pm$ 0.40 mg, and CYP2C9x/x = 1.79 $\pm$ 1.79 mg; P = .0064; Table 2 and Figure 1B).

Potential interactions between CYP2C9 and VKORC1 polymorphisms were also evaluated in relation to warfarin dose requirements with an F test showing significant differences in dosing for CYP2C9 genotypes within the VKORC1-1639G/A subgroup (P = .013). No significant differences were found for CYP2C9 within the VKORC1 GG and AA subgroups (P = .610 and .380, respectively), probably due to the absence of CYP2C9x/x genotypes within these groups (Figure 2).

A stepwise multivariate linear regression model for daily warfarin dose revealed that age, VKORC1-1639G/A, and CYP2C9*1, *2, and *3 polymorphisms explain 20.9%, 11.3%, and 7.1% of interindividual variance, respectively (P = .001; Table 3). Contrarily, gender, BSA, and BMI showed no influence on

Table 3. Multiple Regression Model for Logarithmically Transformed Daily Warfarin Maintenance Dose

Covariates	β Coefficient	SD	*P* Value	R^2 for Model, %
Age (years)	−0.0058	0.0009	<.001	20.90
CYP2C9 genotype	−0.0850	0.0327	.011	11.32
VKORC1 genotype	−0.0869	0.0214	<.001	7.10
Gender	0.0084	0.0389	.828	0.79
BSA	0.1264	0.1207	.297	0.11
BMI	0.0034	0.0046	.470	0.84
Clinical indication	−0.0113	0.0144	.432	5.96
Constant	0.7337	0.1820	<.001	—

NOTES: BMI = body mass index; BSA = body surface area.

warfarin dose and were excluded from the stepwise regression model due to lack of significance (Table 3).

Discussion

Previous reports have suggested a correlation between warfarin dose and bioenvironmental factors such as age, gender, clinical indication, concurrent medication, BSA, and BMI.[28-30] In our population, age was significantly inverse correlated with warfarin dose ($r = -.465$, $P < .001$) showing a decrease 0.56 mg/d/decade, which corroborates what previous studies had found.[31-33] The fact that body metabolism and vitamin K storage decrease with age has been used as an explanation of this phenomenon.[28] We also found that the clinical indication can influence the daily warfarin effective dose as well. In our study, patients affected with AF required a lower warfarin daily dose anticoagulation therapy than patients affected by other thromboembolic conditions ($P = .0185$). Although this variable did not reach significance in the stepwise regression model, others have reported this effect.[34] The lower dose found in AF patients is obvious, and this is attributed to either physicians undertreating such patients (ie, keeping them at a lower target range) or to a higher therapeutic range such as mechanical heart valves or DVT/AT. However, clinicians were blind to patient genotypes, so we estimate that the probability of undertreatment causing any bias regarding the genetic conclusions is low.

The mean warfarin daily dose was significantly lower in patients with the *CYP2C9*2* or *CYP2C9*3* alleles compared to patients with the wild-type allele, as expected for the enzymes with deficient metabolic activity.[13-16] Similarly, the *VKORC1*-1639G/A promoter variant had a significant effect on daily warfarin dose requirements. Homozygous (GG) and heterozygous (GA) patients required higher doses than patients with *AA* genotype.

Overall, age and *VKORC1* and *CYP2C9* genotypes explained 38.3% of the interindividual variation of warfarin maintenance dose (*VKORC1*-1639G/A explained 11.3%; *CYP2C9*1,* *2, and *3, 7.1%; and age, 20.9%). This percentage is low compared to those reported for Caucasians.[11,35] In contrast with Caucasian populations, Kimura et al. found a low contribution of both genes in Asians due to lower frequencies of these polymorphisms in that population.[11,35]

Recently, mutations on *VKORC1* that cause warfarin resistance have been described.[8,36,37] In our study, 30 patients were considered resistant with a mean daily dose of 7.4 mg/d. It is possible that some of these mutations are found in our population, which explains the occurrence of a few cases of extreme requirements. It would be interesting to examine all the patients in the warfarin-resistant group to determine whether this phenotype is caused by some variation of this gene. However, it is expected that only a few patients have such mutations. With the inclusion of a greater number of SNPs on *VKORC1*, a higher percentage of explained variability could likely be reached. It is also important to consider rare mutations and/or polymorphisms on *VKORC1* and *CYP2C9* that could have an effect on warfarin dose and that could be specific to our population, as well as variants of other genes coding for proteins involved in clot formation.

It is worth mentioning that people were prospectively recruited without our previous knowledge of the final outcome that would allow for group assignment. Once each patient was recruited and evaluated, we calculated post hoc the 25th and 75th percentiles of mean daily doses to identify sensitive, intermediate, and resistant groups of patients. This recruitment method and categorical definition do not introduce any bias as to increasing the probabilities of finding significant genetic effects. One limitation might be the inclusion of patients with a stable therapeutic INR, which introduces a bias because the influence

of different predictors on the warfarin dose requirement could differ between these patients and those for whom it proves impossible to achieve a stable dose. In the same vein, we found differences in our outcomes from other similar studies testing factors that influence warfarin dose (such as BSA, BMI, and gender). In our study, these factors failed to produce any significant differences. Variables associated to both effect and sample size might be responsible for this result.

Even though the medical literature contains hundreds of manuscripts concerned with the pharmacogenetics of warfarin response, we found only 1 manuscript investigating the effects of *CYP2C9* and *VKORC1* in Hispanic populations. Furthermore, only 6 manuscripts describe genetic interactions between these 2 genes. The model of interaction among bioenvironmental variables (age) and genetic variants harbored at these 2 genes provides a contrastable instrument to study dose requirements in populations of Hispanic heritage. It is worth mentioning that the Hispanic population in the United States represents the biggest minority group ($\sim$40 million people). We strongly believe that this study provides new insights into the gene-bioenvironment interaction outlining the response to anticoagulant treatment with warfarin. Our data provide important implications for populations with predominant Iberian ancestry such as Colombia and the self-designated Paisa community, which originated a postcolonial genetic and cultural isolate that is currently disseminated throughout the western part of the country. This is an interesting illustration to encourage pharmacogenetic typification in Latin American regions or countries that have had different geographic patterns of genetic admixture and different admixture dynamics.

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