A polymorphism in the *VKORC1* gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin

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Patients require different warfarin dosages to achieve the target therapeutic anticoagulation. The variability is largely genetically determined, and it can be only partly explained by genetic variability in the cytochrome CYP2C9 locus. In 147 patients followed from the start of anticoagulation with warfarin, we have investigated whether *VKORC1* gene mutations have affected doses of drug prescribed to acquire the target anticoagulation intensity. Two synonymous mutations, 129C>T at Cys43 and 3462C>T at Leu120, and 2 missense mutations, Asp38Tyr and Arg151Gln, were identified. None of these mutations was found to affect the interindividual variability of warfarin prescribed. Finally, 2 common polymorphisms were found, 1173C>T in the intron 1 and 3730G>A transition in the 3′ untranslated region (UTR). Regardless of the presence of confounding variables, the mean adjusted dose required of warfarin was higher (6.2 mg) among patients with the *VKORC1* 1173CC genotype than those of patients carrying the CT (4.8 mg; *P* = .002) or the TT genotype (3.5 mg; *P* < .001). In the present setting, *VKORC1* and CYP2C9 genetic variants investigated accounted for about a third (r^2, 0.353) of the interindividual variability. Genetic variants of the *VKORC1* gene locus modulate the mean daily dose of drug prescribed to acquire the target anticoagulation intensity. (Blood. 2005;105:645-649)

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Introduction

Oral anticoagulation for the prevention and treatment of patients with arterial and venous thromboembolic disorders is one of the most used therapies in clinical practice. Bleeding is by far the most important complication of oral anticoagulation.[1-5] Much effort has been devoted to improve the safety of oral anticoagulation. However, the bleeding risk remains significantly high.[5,6]

Warfarin is the widespread oral anticoagulant drug used, and the required dose is variable, in particular between individuals but also within 1 individual, and depends on several factors (eg, dietary intake, variations in pharmacokinetics and pharmacodynamics, compliance, etc).[7] Besides acquired and environmental factors, it is well known that the response to warfarin is largely genetically determined.[8]

The cytochrome P450 CYP2C9 is a liver enzyme required for the oxidative metabolism of a large number of clinically important drugs, including warfarin.[9] A series of genetic polymorphisms have been described within the cytochrome P450 CYP2C9 locus.[10] Two gene variants, a substitution of a cysteine for an arginine at position 144 within the exon 3 (CYP2C9*2) and a substitution of a leucine for an isoleucine at position 359 within the exon 7 (CYP2C9*3), have been shown to impair hydroxylation of warfarin in vitro.[11,12] These allelic variants of CYP2C9, code for enzymes with approximately 12% (CYP2C9*2) and 5% (CYP2C9*3) of the enzymatic activity of the wild-type genotype CYP2C9*1.[11,13,14] Both variant alleles have been associated with decreased warfarin dose requirements, more time to achieve stable dosing, a higher risk of bleeding during the initiation phase, and a significantly higher bleeding rate.[15-23]

However, allelic variants of CYP2C9 do not explain the large interindividual variability in the dose-anticoagulant effect of warfarin, suggesting that additional factor(s) contribute to this variability. Very recently, by using a positional cloning approach a novel gene responsible, at least in part, for the activity of the vitamin K epoxide reductase (VKOR) complex, the vitamin K epoxide reductase complex subunit 1 *(VKORC1)* gene, has been identified.[24,25] Four different heterozygous missense mutations have been found in probands suffering from warfarin resistance.[24]

In a cohort of warfarin-anticoagulated patients followed up at 1 specialized anticoagulant clinic from the start of treatment, we have investigated the influence of variants of the *VKORC1* gene on the mean daily dose of drug prescribed to acquire the target anticoagulation intensity.

Patients, materials, and methods

After approval of the local ethics committee, the study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from all participants.

Patients

The cohort was previously investigated for the effect of CYP2C9 allelic variants on dose requirement of warfarin.[18] Of the 180 subjects initially investigated for that study, DNA samples were available in 147. Full details

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of the study design and recruitment criteria are presented elsewhere.[18] Caucasian patients, who were prescribed oral anticoagulation, from May 1995 to April 1999, were recruited from the Coagulation Centre of the "A. Cardarelli Hospital," Naples. This center belongs to the Italian Federation of Anticoagulation Clinics, which requires each center to give extensive instructions to all new patients enrolled; follow patients by International Normalized Ratio (INR); fix the date for the next visit and prescribe daily anticoagulant dose; monitor changes in patients habits, diet, and co-medication, illnesses, bleeding complications, and scheduled surgical or invasive procedures; take part in external laboratory quality control. Outpatients that attended the center during the study period, June 1, 1995, to June 30, 1999, were requested to enter into the study.

A complete clinical summary was obtained from all subjects by a specially trained staff. All records from visits to the center were reviewed. The follow-up period considered for clinical end points started the day on which anticoagulation began and ended on the day when sampling for DNA analysis occurred.

Seventy-seven apparently healthy subjects (35 men and 42 women; median age, 52.5 years; range, 31-73) randomly selected from a Southern Italian general population served as control subjects.

DNA extraction and analysis

DNA was extracted from peripheral blood leukocytes according to standard protocols.[18] Genotyping of the CYP2C9 alleles was detected as previously described.[18] Amplifications of all coding regions of *VKORC1* gene and intron/exon boundaries, 718 base pair (bp) of the 5′ untranslated region (UTR) and 404 bp of the 3′ UTR were achieved using sense and antisense oligonucleotide designed and numbered on the basis of known sequences of *VKORC1* gene locus (GenBank accession no. AY587020; the A of the ATG initiation codon is denoted nucleotide +1) (Table 1). Oligonucleotide custom synthesis service was from Life Technologies (Paisley, United Kingdom). PCR was carried out on 50-μL volume samples, in a Perkin Elmer-Cetus thermal cycler (Perkin-Elmer Cetus, Norwalk, CN). Each sample contained 0.1 μg genomic DNA, 10 pmol of each primer, 125 μM deoxyribonucleoside triphosphate (dNTP), 5 mM tris(hydroxymethyl)aminomethane (Tris) HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U Taq polymerase. Then amplified DNA fragments were subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer sequencer (PE Biosystems, Foster City, CA).

RNA analysis

To study the effect on the mRNA of the *VKORC1* 1173C>T polymorphism identified, a 3722-bp construct with the C or the T allele was made containing all exons and intervening sequences, 49 bp of the 5′ UTR, and 67 bp of the 3′ UTR.

For both alleles, all PCR-derived constructs were sequenced to determine whether there were any PCR-induced mutations. A final step was at 72°C for 5 minutes to ensure a 3′ adenylated PCR product was added. Before cloning, the PCR product was purified from a 1.0% agarose gel by Concert Rapid Gel Extraction System (Life Technologies) according to manufacturer's instructions. The purified PCR product was cloned using Eucariotic TA Expression Kit Bidiretional (Invitrogen, Groningen, the Netherlands) according to manufacturer's instructions.

Plasmid DNA was purified by Pure Plasmid Isolation Kit (Roche, Indianapolis, IN), and positive clones were sequenced in an ABI PRISM 3100 Genetic Analyzer to identify clones showing the correct 5′ → 3′ orientation. Clones that showed the correct 5′ → 3′ orientation were transfected in HELA cells. Briefly, HELA cells were grown in 10% fetal bovine serum/Roswell Park Memorial Institute (FBS/RPMI) medium (Life Technologies). One day before the transfection, cells were seeded in 6-well plates (400 000 cells/well). The transfection was performed using the FuGENE 6 Transfection Reagent kit (Roche) according to manufacturer's instructions. Total RNA was purified by TRIzol Reagents (Life Technologies) according to manufacturer's instructions. Reverse transcription was made by Reverse Transcription System Kit (Promega, Madison, WI). The mRNA splicing product was purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer sequencer (PE Biosystems) according to the manufacturer's instructions.

Statistical analysis

For each patient the average daily dose of warfarin (in mg) prescribed and the average INR were obtained. The average daily dose of warfarin prescribed was calculated sharing the sum of warfarin prescribed by the time (in days) in anticoagulation. The average INR was obtained summing all INR available and then sharing by the number of visits. The allelic frequencies were estimated by gene counting, and genotypes and haplotypes were scored. Differences in baseline characteristics among genotypes were evaluated by the Mann-Whitney *U* test and chi-square test for continuous and discrete variables, respectively. Multiple comparisons among different CYP2C9 haplotypes were made using univariate analysis of variance (ANOVA). Pairwise multiple comparisons were performed using Scheffé test. Multiple linear regression models, adjusted for age when oral anticoagulation started, sex, indication, average INR, time (in years) on oral anticoagulation, and number of visits were used to investigate the influence of *VKORC1* genotypes and CYP2C9 haplotypes on average daily dose of warfarin prescribed. The effect of other drugs prescribed was investigated in different models adjusted for the total number of drugs coadministered or only for drugs known to be metabolized by the cytochrome CYP2C9. Drugs affecting the cytochrome CYP2C9 found in prescriptions of patients analyzed were amitriptyline, barbiturates, carbamazepine, diclofenac, disulfiram, glipizide, fluvastatin, ketoprofen, ibuprofen, lovastatin, phenylbutazone, phenytoin, piroxicam, and sulfamethoxazole-trimethoprim. General factorial ANOVA models, adjusted for the same variables, were used to investigate the possibility of an interaction between different genotypes/haplotypes investigated on average daily dose of warfarin prescribed. All the analyses were performed according to the Statistical Package for Social Science (SPSS 10.0 for Macintosh; SPSS Science, Chicago, IL). A 5% 2-tailed significance level was used for all tests.

Table 1. Primers used for amplification and sequencing of the *VKORC1* gene, fragment size, and polymerase chain reaction (PCR) conditions

Primer	Position	Sequence	Amplicon size	Annealing temperature
VKORC1 5′ UTR FW	(−)718 to (−)699	TGATCCGCTGGTCTCTAGGT	786 bp	59°C
VKORC1 5′ UTR REV	68-49	GAGAGCACTAAGCCCGTCAG		
VKORC1 EX1 FW	(−)49 to (−)31	CTCCGTGGCTGGTTTTCTC	303 bp	57°C
VKORC1 EX1 REV	254-235	CCGATCCCAGACTCCAGAAT		
VKORC1 EX2 FW	1120-1139	TGACATGGAATCCTGACGTG	361 bp	57°C
VKORC1 EX2 REV	1480-1463	GAGCTGACCAAGGGGGAT		
VKORC1 EX3 FW	3348-3365	AGTGCCTGAAGCCCACAC	326 bp	57°C
VKORC1 EX3 REV	3673-3654	ACCCAGATATGCCCCCTTAG		
VKORC1 3′ UTR FW	3534-3553	AGCCTGATGTGGCTCAGTTT	467 bp	58°C
VKORC1 3′ UTR REV	4000-3981	ATAACCACCCTTAAACGCAG		

Oligonucleotides are numbered according to the AY587020 sequence, the A of the ATG initiation codon is denoted as the nucleotide +1.

Results

Patient characteristics and oral anticoagulation

Of a total of 203 patients who attended the clinic during the study period, 199 were prescribed warfarin and followed up for 75 days or more, and were invited to take part in the study. Fifteen individuals refused consent and in another 37 patients genotyping was unavailable for technical reasons. Actually, the DNA was not obtained in 4 individuals, terminated since used in previous studies in 21, and not sufficient for all analyses made in the present investigation in 12. Thus, 147 patients (median age, 43.0 years; range, 15-84) were analyzed. Male sex, indication, mean duration of the oral anticoagulation, average dose of warfarin received, average INR, and number of visits were not significantly different in patients included as compared with those not analyzed (data not shown).

Analysis of the *VKORC1* gene locus

In all subjects, the *VKORC1* gene was investigated for gene variants by means of direct cycle sequence analysis. The sequencing of the entire coding region and exon-intron boundaries revealed 2 missense mutations. The first variation detected was a heterozygous aspartate to tyrosine (112G>T) substitution at position 38 that occurred in the exon 1. The second missense mutation, a heterozygous Arg-to-Gln substitution (3556G>A) at the residue 151, was identified in the exon 3. Each mutation was found in 1 subject. In addition, 2 synonymous mutations, a 129C>T transition at Cys43 in the exon 1 and a 3462C>T transition at Leu120 in the exon 3 (rs7200749; see www.ncbi.nlm.nih.gov/SNP/snp.ref.cgi?-locusld = 79001) were identified in 1 and 2 individuals, respectively. Then, 2 polymorphisms were identified in the portion of the intron 1 analyzed, a C>T transition at position 1173 (rs9934438), and in the 3′ UTR, a 3730G>A transition (rs7294). Allele and genotype frequencies of polymorphisms identified are reported in Table 2. In the entire setting, 54 patients (36.8%; 95% confidence interval [CI], 28.9-44.5) carried the C allele at the nucleotide 1173, whereas 69 (46.9%; 95% CI, 42.8-51.0) were heterozygotes and 24 individuals (16.3%; 95% CI, 10.3-22.3) carried the T allele (Table 2). Sixty-seven patients (45.5%; 95% CI, 37.4-53.6) showed the 3730G allele, 58 (39.5%; 95% CI, 31.6-47.4) were heterozygotes, and 22 (15.0%; 95% CI, 9.2-20.8) carried the A allele (Table 2). Genotype distribution and allele frequencies for the 1173C>T transition were evaluated in 77 apparently healthy subjects. No significant difference was observed between the normal population and individuals requiring oral anticoagulation. Actually, 20 subjects (26.0%) carried the C allele, 45 (58.4%) were heterozygotes, and 12 individuals (15.6%) carried the T allele. The calculated frequency of the C allele was 55.2%, whereas that of the T allele was 44.8%.

Table 2. Genetic characteristics of anticoagulated patients

Polymorphism	Allele	N (%)	Genotype	N (%)
VKORC1				
1173C>T	C	177 (60.2)	CC	54 (36.8)
	T	117 (39.8)	CT	69 (46.9)
			TT	24 (16.3)
3730G>A	G	192 (65.3)	GG	67 (45.5)
	A	102 (34.7)	GA	58 (39.5)
			AA	22 (15.0)
CYP2C9				
Arg144Cys	Arg	244 (83.0)	Arg/Arg	97 (66.0)
	Cys	50 (17.0)	Arg/Cys	50 (34.0)
			Cys/Cys	—
Ile359Leu	Ile	268 (91.2)	Ile/Ile	122 (83.0)
	Leu	26 (8.8)	Leu/Ile	24 (16.3)
			Leu/Leu	1 (0.7)

– indicates ______________.

CYP2C9 haplotypes

As the *CYP2C9* gene is concerned (Table 2), 48 patients (32.7%; 95% CI, 25.6-40.3) carried a cysteine at position 144 within the exon 3, all heterozygotes, and an isoleucine at position 359 within the exon 7 (CYP2C9*2 haplotype). Twenty-three patients (15.7%; 95% CI, 9.8-21.6), 22 heterozygotes, and 1 homozygote showed an arginine at position 144 within the exon 3 and a leucine at position 359 within the exon 7 (CYP2C9*3 haplotype). Two patients carried both a cysteine at position 144 within the exon 3 and a leucine at position 359 within the exon 7 (CYP2C9*2 + CYP2C9*3 haplotype).

Phenotype analysis

The subject carrying the *VKORC1* Asp38Tyr mutation was a woman who at the age of 54 years suffered from a proximal deep vein thrombosis in the right leg and pulmonary embolism. The average daily dose of warfarin prescribed was 5.3 mg and the mean INR value was 2.56. The subject carrying the *VKORC1* Asp151Gln mutation was a man who suffered from a deep vein thrombosis in the left leg at the age of 57 years and was taking, at the time of the sampling, an average daily dose of warfarin of 3.7 mg. His mean INR value was 2.54. Main characteristics of different 1173C>T and 3730G>A genotypes are shown in Table 3. Male sex, indication, mean duration of the oral anticoagulation, averages of the regular estimations of the INR, percentage of individuals taking other drugs, and number of visits were not significantly different among groups with different *VKORC1* (1173C>T, 3730G>A) genotypes and CYP2C9 haplotypes. Age when the oral anticoagulation started was significantly higher in the 2 patients with the CYP2C9*2 + CYP2C9*3 haplotype, when compared with those carrying the CYP2C9*1 or the CYP2C9*2 haplotype ($P < .05$, Scheffé test).

The average dose of warfarin received from each patient was higher among patients with the *VKORC1* 1173CC genotype (7.0 mg) than that of patients carrying the CT (5.1 mg; $P < .001$, Scheffé test) or the TT genotype (3.7 mg; $P < .001$, Scheffé test). In addition, a trend was observed when comparing the mean daily dose required in patients carrying the CT with that of patients carrying the TT genotype ($P = .064$, Scheffé test). Subjects with the 3730AA genotype were prescribed more warfarin (6.9 mg) than patients carrying the GG (5.2 mg; $P < .05$, Scheffé test) or the GA genotype (5.3 mg; $P = .065$, Scheffé test). Anticoagulated patients carrying the CYP2C9*1 haplotype were prescribed a higher mean dose (6.6 mg) than patients with the CYP2C9*2 (5.1 mg; $P < .05$, Scheffé test) or the CYP2C9*3 haplotype (3.5 mg; $P < .05$, Scheffé test; Table 3). The dose was further lower in the 2 patients with the CYP2C9*2 + CYP2C9*3 haplotype (1.8 mg).

The possibility that different genotypes modulate dose requirements of warfarin was further investigated in a multiple linear regression model, adjusted for age when oral anticoagulation started, sex, indication, average INR, time (in years) on oral anticoagulation, other drugs prescribed, and number of visits. A stepwise analysis revealed the independent nature of the *VKORC1* 1173C>T polymorphism (r^2, 0.138; $P < .001$) with respect to the average dose of warfarin received. As expected, gene variants within the CYP2C9 locus significantly affected the average dose of warfarin prescribed (r^2, 0.215; $P < .001$). In

Table 3. Clinical characteristics of patients carrying different genotypes

	Sex, (m/f)	Indication, v/a/o	Mean age when OAT started, y (SD)	Mean time in OAT, y (SD)	Warfarin mean daily dose, mg (SD)	Mean INR (SD)	Patients taking other drugs, % (n)	Visits, n (SD)
VKORC1								
1173C>T								
CC, n = 54, 36.8%	32/22	42/8/4	42.8 (16.5)	1.9 (2.9)	7.0 (3.0)*	2.45 (0.39)	35.2 (19)	28.4 (24.0)
CT, n = 69, 46.9%	34/35	55/8/6	43.4 (16.0)	1.5 (1.4)	5.1 (2.5)*	2.56 (0.39)	37.7 (26)	27.3 (23.7)
TT, n = 24, 16.3%	14/10	15/5/4	49.6 (18.4)	1.3 (1.2)	3.7 (1.6)	2.53 (0.37)	45.8 (11)	25.2 (20.1)
3730G>A								
GG, n = 67, 45.5%	31/36	49/11/7	44.2 (18.0)	1.5 (1.3)	5.2 (2.6)†	2.52 (0.35)	42.7 (23)	26.6 (21.9)
GA, n = 58, 39.5%	35/23	42/11/5	46.2 (15.1)	1.7 (2.8)	5.3 (2.2)	2.60 (0.45)	24.3 (25)	25.5 (21.6)
AA, n = 22, 15.0%	14/8	21/1/0	44.3 (16.5)	1.6 (1.6)	6.9 (4.0)	2.38 (0.31)	36.6 (7)	28.9 (26.6)
CYP2C9								
Allele*1, n = 74, 50.3%	44/30	56/11/7	41.9 (15.3)‡	1.8 (2.5)	6.6 (2.9)	2.47 (0.36)	36.5 (27)	27.7 (22.3)
Allele*2, n = 48, 32.0%	25/23	37/6/5	44.5 (17.6)	1.7 (1.5)	5.1 (2.2)§	2.58 (0.41)	37.5 (18)	28.2 (23.2)
Allele*3, n = 23, 16.3%	11/12	18/4/1	47.9 (16.7)	1.2 (1.3)	3.5 (1.9)§	2.63 (0.44)	39.1 (9)	22.3 (22.2)
Allele*2 + Allele*3, n = 2, 1.4%	0/2	1/0/1	76.5 (9.2)	2.3 (2.7)	1.8 (0.1)§	3.00 (0.21)	100.0 (2)	55.0 (58.0)

v/a/o indicates patients with previous venous (v) or arterial (a) thrombosis, or other (o) disease requiring oral anticoagulation; OAT, oral anticoagulant therapy.

*$P < .001$ vs TT carriers (Scheffé test).

†$P < .05$ vs AA carriers (Mann-Whitney U test).

‡$P < .05$ vs CYP2C9*2 + CYP2C9*3 carriers (Scheffé test).

§$P < .05$ vs CYP2C9*1 carriers (Scheffé test).

addition, a significant effect was exerted by the age when oral anticoagulation started (r^2, 0.063; $P < .001$).

A general factorial ANOVA model, which included the same variables, was performed to address the possibility of an interaction between significant variables. The analysis confirmed the association with the *VKORC1* 1173C>T polymorphism (F, 8.585; $P \leq .001$) and the CYP2C9 haplotype (F, 8.774; $P < .001$). The adjusted dose required of warfarin was significantly higher in patients carrying the *VKORC1* 1173CC genotype or the CYP2C9*1 haplotype (Table 4). No significant interaction between any of the variables analyzed was found (data not shown).

Effect of the *VKORC1* 1173C>T polymorphism on *VKORC1* mRNA splicing

To determine whether the 1173C>T polymorphism could affect the processing of the *VKORC1* gene primary transcript, HELA cells were transfected with a construct containing the C or the T allele, all exons and introns, and portions of the 5′ UTR and 3′ UTR (see "Patients, materials, and methods"). HELA cells containing the 1173C allele displayed a band of approximately 700 bp (not shown), which corresponded fairly well to the expected splicing product of 676 bp. Likewise, in HELA cells transfected with the 1173T construct a band of approximately 700 bp was present (not shown). This suggested that the splicing product observed was consistent with a normal mRNA processing. To confirm that the 1173C>T transition did not cause an abnormal mRNA processing, the band observed in HELA cells transfected with either construct was purified and then sequenced. In both cases, the expected sequence was observed (not shown), thus confirming that the 1173C>T polymorphism does not affect *VKORC1* mRNA processing.

Table 4. Adjusted mean doses of warfarin prescribed according to different genotype/haplotype

	Adjusted mean, mg	95% CI	Significance
VKORC1			
1173CC	6.2	5.0-7.3	Ref
1173CT	4.8	3.8-5.9	.002
1173TT	3.5	2.2-4.8	< .001
			.018*
CYP2C9			
CYP2C9*1	6.0	5.3-6.7	Ref
CYP2C9*2	4.7	3.8-5.5	.002
CYP2C9*3	3.8	2.7-4.8	< .001
CYP2C9*2 + CYP2C9*3	4.9	1.6-8.3	NS

Adjusted mean is calculated as the mean daily dose of warfarin taking into account other variables.

CI indicates confidence intervals; Ref, reference group, NS, not significant.

*Versus CT genotype.

Discussion

The oral anticoagulant therapy is widely used for the management and the prevention of venous and arterial thrombosis. The use of the same fixed dose of warfarin for all patients is unachievable because the responsiveness of different patients to warfarin is highly variable. For this reason, the anticoagulant effect of warfarin is measured by a standardized prothrombin time, and the dose is adjusted accordingly.[26] Gene variants of the *CYP2C9* gene locus, which encodes for an enzyme that metabolizes warfarin, have been associated with large interindividual differences on the anticoagulant response to warfarin.[15-23] However, also taking into account the CYP2C9 haplotypes a wide variability among subjects assuming warfarin remains, suggesting that additional factor(s) contribute to this variability. Very recently, genetic variations within the gene encoding for a subunit of the vitamin K epoxide reductase complex, namely the *VKORC1* gene, have been found to predict sensitivity to warfarin therapy.[24] In a cohort of patients followed from the start of the anticoagulation in 1 clinic, which belongs to and fulfills instructions of the Italian Federation of Anticoagulation Clinics, we have investigated whether common and sporadic gene variants at the *VKORC1* gene locus significantly contribute to the interindividual variability to warfarin therapy. To our knowledge, this is the first study that has searched for this relationship in individuals without warfarin resistance. The *VKORC1* gene locus spans about 5 Kbp, encompassing 3 exons and 2 introns, and encodes for a small protein of 163 residues. In the present study, 2 synonymous mutations already reported were identified.[24] In addition, 2 missense mutations, Asp38Tyr and Arg151Gln, occurring in the *VKORC1* exon 1 and 3, respectively, were found. Both substitutions involved residues not conserved among human, mouse,

and rat sequences. In keeping with this, none of these mutations was found to affect the interindividual variability in the average daily dose of warfarin prescribed. Finally, 2 common polymorphisms were found in the intron 1, the 1173C>T transition, and in the 3′ UTR, the 3730G>A transition.

Carriers of the 1173TT genotype had assumed a dose of warfarin significantly lower than those of carriers of the CC genotype or patients with the CT genotype. Patients with the different genotypes did not differ for sex, indication and duration of anticoagulation, age when anticoagulation started, and average INR. On the other hand, the 3730G>A polymorphism was associated with differences in the average dose of warfarin prescribed, patients carrying the GG genotype showing a significant lower average daily dose of warfarin prescribed. As expected, carriers of the CYP2C9*2 and CYP2C9*3 haplotypes had assumed a mean dose of warfarin significantly lower than those of carriers of the CYP2C9*1 haplotype. Patients with the different CYP2C9 haplotypes did not differ for sex, indication and duration of anticoagulation, number of visits, and average INR.

The dose of warfarin prescribed is strictly related to the intensity of anticoagulation required and a series of confounding variables, as the interference of diet habits or the contemporary prescription of drugs, may affect interindividual variability. As a rule, the more drugs a patient receives concomitantly with warfarin, the more difficult is the control of anticoagulation. There is a strong correlation between total number of drugs prescribed for a patient and the likelihood that a patient will show effects of an interacting drug.[27] To adjust for these confounding variables, average INR, the mean of all INRs recorded when a patient attended a prescheduled clinic visit, and the contemporary prescription of additional drugs known to interact with the cytochrome CYP2C9 were also considered in multivariate analyses. Both the 2 statistical models applied, multiple linear regression and general factorial ANOVA, confirmed that the *VKORC1* 1173C>T polymorphism, and CYP2C9 haplotypes, significantly allowed for different average doses of warfarin prescribed to attain the prescheduled anticoagulation target. No relationship was found with the other *VKORC1* polymorphism investigated. All together, genetic variants investigated accounted for about a third (r^2, 0.353) of the interindividual variability calculated in the present setting.

The biologic mechanism by which variants in the *CYP2C9* gene are associated with large interindividual pharmacokinetic and pharmacodynamic differences in the outcome of warfarin therapy is well-known in individuals with *CYP2C9* gene variants showing a reduced metabolic capacity.[28] Possible explanations for the significant association of *VKORC1* 1173C>T polymorphism with the average daily dose of warfarin prescribed found in the present report are matters of hypothesis. We did not find any effect of the 1173C>T polymorphism in selecting splice sites of *VKORC1* mRNA. Thus, we exclude the possibility that different alleles are related to an alternative mRNA splicing of *VKORC1* gene. Other possible explanations to findings obtained in the present cohort have to be taken into consideration, as it is conceivable that the *VKORC1* 1173C>T polymorphism is in linkage disequilibrium with unknown allelic variants that modulate sensitivity to warfarin therapy.

In conclusion, we first described that polymorphisms in a gene locus encoding for a subunit of the vitamin K epoxide reductase complex, the *VKORC1* gene, may play a significant role in the modulation of the anticoagulant effect of the dose of warfarin prescribed. Whether screening for these polymorphisms identifies patients needing anticoagulation regimens of different intensities is beyond the aims of this study and deserves to be addressed.

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