Thrombosis • Research Paper

Polymorphisms in factor II and factor VII genes modulate oral anticoagulation with warfarin

[haematologica]
2004;89:1510-1516

ROSA LUCIA D'AMBROSIO
GIOVANNA D'ANDREA
FILOMENA CAPPUCCI
MASSIMILIANO CHETTA
PASQUALE DI PERNA
VINCENZO BRANCACCIO
ELVIRA GRANDONE
MAURIZIO MARGAGLIONE

A B S T R A C T

Background and Objectives. There is very considerable inter-individual variability in warfarin dosages necessary to achieve target therapeutic anticoagulation. The variability is largely genetically determined but can only partly be explained by genetic variability in the cytochrome *CYP2C9* locus. Polymorphisms within the genes coding for vitamin K-dependent proteins have been suggested to predict sensitivity to warfarin therapy.

Design and Methods. In a cohort of 147 patients followed-up at one specialized clinic from the start of anticoagulation with warfarin, we investigated whether factor II (Thr165Met; G20210A) and factor VII polymorphisms (G-402A; G-401T) affected the doses of warfarin necessary to acquire the target intensity of anticoagulation.

Results. Regardless of the presence of confounding variables, the mean adjusted dose of warfarin required was higher among patients with the factor II Thr/Thr 165 genotype (4.2 mg) than among patients carrying the Met165 allele (2.9 mg; p=0.041) and higher in carriers of the factor VII GG-401 genotype (4.1 mg) than in those with the T-401 allele (3.1 mg; p=0.029). No significant effect was found for factor II A20210G and factor VII G-402A polymorphisms. All together, the genetic variants investigated accounted for about a quarter (r2: 0.261) of the inter-individual variability calculated in the present setting.

Interpretation and Conclusions. Genetic variants of factor II and factor VII modulate the mean daily dose of warfarin required to achieve a target intensity of anticoagulation.

Key words: warfarin, factor II, factor VII, polymorphisms, dose requirement.

*From the #Istituto di Genetica Medica, Dipartimento di Scienze Biomediche, Università di Foggia, *Unità di Aterosclerosi e Trombosi, I.R.C.C.S. "Casa Sollievo della Sofferenza", S.0 Giovanni Rotondo, and †Divisione di Ematologia, Unità di Coagulazione, Ospedale "A. Cardarelli," Naples, Italy.*

Corresponcence:
Maurizio Margaglione, MD,
Istituto di Genetica Medica,
Dipartimento di Scienze Biomediche, Università di Foggia,
viale Pinto, 71100 Foggia, Italy.
E-mail: m.margaglione@unifg.it

Oral anticoagulation for the prevention and treatment of patients with arterial and venous thromboembolic disorders is one of the most widely employed therapies in clinical practice. A long period of anticoagulation may be helpful,[1,2] particularly in cases in which venous thrombosis is not associated with a reversible risk factor.[3] Bleeding is by far the most important complication of the oral anticoagulation.[4-8] Much effort has been devoted to improving the safety of oral anticoagulation by adopting a series of measures (international normalized ratio [INR], lower anticoagulation levels, etc.), and these have reduced the rates of major and minor hemorrhages. Nevertheless, the risk of bleeding still remains high.[8,9] Many studies have been conducted to identify high-risk patients.[4-9] The overall incidence of bleeding episodes was found to be lower among individuals aged 70 years or less and in patients receiving lower intensity regimens of oral anticoagulation.[8] Life-threatening and fatal bleeding complications occurred more often in elderly patients.[9]

Warfarin is the most widely used oral anticoagulant; the dose required varies between individuals but also within the same individual, and depends on several factors, e.g. dietary intake, variations in pharmacokinetics and pharmacodynamics, and compliance.[10] Beside acquired and environmental influences, it is well known that the variable response to warfarin has a large genetic component.[11] Cytochrome P450 is a liver enzyme required for the oxidative metabolism of a large number of clinically important drugs, including warfarin.[12] The gene coding for this enzyme is *CYP2C9* and a series of genetic polymor-

phisms have been described within the *CYP2C9* locus.[13] Two gene variants of *CYP2C9*, coding for enzymes with approximately 12% (*CYP2C9*2*) and 5% (*CYP2C9*3*) of the enzymatic activity of the wild-type genotype (*CYP2C9*1*),[14-16] have been shown to impair hydroxylation of warfarin *in vitro*.[14-17] 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 rate of bleeding.[18-26]

However, allelic variants of *CYP2C9* do not explain the large inter-individual variability in the dose-anticoagulant effect of warfarin suggesting that additional factor(s) contribute to this variability. Very recently, polymorphisms of the genes encoding vitamin K-dependent proteins have been found to contribute to warfarin sensitivity.[27]

In a cohort of warfarin-anticoagulated patients followed-up at one specialized anticoagulant clinic from the start of treatment, we investigated the influence of variants of factor II and factor VII on the mean daily dose of drug required in order to reach the target intensity of anticoagulation.

Design and Methods

After approval from 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 had been previously investigated for the effect of *CYP2C9* allelic variants on dose requirement of warfarin.[21] Of the 180 Caucasian subjects initially investigated in that study, sufficient DNA was available from 147 for this study. Full details of the study design and recruitment criteria are presented elsewhere. Caucasian patients who were prescribed oral anticoagulation from May 1995 to April 1999 were recruited from the Coagulation Center of the *A. Cardarelli* Hospital, Naples. This Center is part of the Italian Federation of Anticoagulation Clinics, which requires each Center to: give extensive instructions to all new patients enrolled; follow-up patients by regular International Normalized Ratio (INR) measurements; fix the date for the patients' next appointment and prescribe a daily anticoagulant dose; monitor changes in patients' habits, diet, and co-medication, illnesses, bleeding complications and scheduled surgical or invasive procedures; and take part in external laboratory quality control. Outpatients who attended the Center during the study period, June 1, 1995 to June 30, 1999, were asked to participate in the study.

A complete clinical summary was obtained from all subjects by specially trained staff. All records from visits to the Center were reviewed. The follow-up period considered started the day on which anticoagulation began and ended on the day when the last visit occurred.

Factor II (FII) coagulant assay. A thromboplastin-based assay using factor II deficient plasma (Diagnostica Stago) was employed to measure factor II (prothrombin) activity in 101 apparently healthy subjects (54 men and 47 women) randomly selected from a Southern Italian population. Measured levels of circulating FII were expressed as a percentage of the amount of FII in pooled normal plasma (arbitrarily designated as 100%). Clotting assays were performed on a KC4 Amelung coagulometer (Amelung, Germany).

DNA extraction and analysis. DNA was extracted from peripheral blood leukocytes according to standard protocols.[21] The *CYP2C9* alleles were genotyped as previously described.[21] The factor II Met165Thr polymorphism was investigated by means of polymerase chain reaction (PCR) and subsequent Nla III restriction enzyme analysis using forward and reverse primers. PCR was carried out on 50 μL samples, in a Perkin Elmer-Cetus thermal cycler. Each sample contained 0.5 μg of genomic DNA, 15 pmoles of each primer, 100 mM of dNTP, 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 1 U thermostable Taq polymerase. The 30 cycles consisted of steps at 95°C for 1 min, at 60°C for 50 sec and at 72°C for 1.5 min. Then, 15 μL of the amplification products were digested for 5 hrs at 37°C with 2 U of the Sml I restriction enzyme. The fragments were fractionated by 3.0% agarose-gel electrophoresis, and visualized under UV light. The G20210A mutation of the factor II gene was assayed as described by Poort *et al.*[28] Genotypes of the G-402A and G-401T polymorphisms in the factor VII promoter were evaluated using sense and antisense oligonucleotides designed on the basis of the known sequence of the FVII gene locus (GenBank accession number J02933). Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Statistical analysis

For each patient the average daily dose of warfarin (mg) prescribed and the average INR were recorded. The average daily dose of warfarin prescribed was calculated by dividing the sum of warfarin prescribed by the time (in days) of anticoagulation. The average INR was obtained by summing all the INR results available and then dividing 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

Table 1. Genetic characteristics of the anticoagulated patients.

Factor II				*Factor VII*				*CYP2C9*			
Polymorphism	*Genotype*	*N*	*%*	*Polymorphism*	*Genotype*	*N*	*%*	*Polymorphism*	*Genotype*	*N*	*%*
Thr165Met	Thr/Thr	132	89.8	G-402A	GG	110	74.8	Arg144Cys	Arg/Arg	98	66.7
	Thr/Met	14	9.5	GA	35	23.8		Arg/Cys	49	33.3	
	Met/Met	1	0.7	AA	2	1.4		Cys/Cys	--		
G20210A	GG	122	83.0	G-401T	GG	98	66.7	Ile359Leu	Ile/Ile	121	82.3
	GA	25	17.0	GT	44	29.9		Leu/Ile	24	16.3	
	AA	–	–	TT	5	3.4		Leu/Leu	2	1.4	

the Mann-Whitney U-test and χ^2 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's test. Multiple linear regression models, adjusted for age at initiation of oral anticoagulation, sex, indication for anticoagulation, average INR, time (in years) on oral anticoagulation, number of visits and of other drugs prescribed, known to be metabolized by *CYP2C9*, were used to investigate the influence of factor II and of factor VII genotypes and *CYP2C9* haplotypes on average daily dose of warfarin prescribed. Drugs affecting *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 obtain adjusted means and to investigate the possibility of an interaction between different genotypes/haplotypes investigated and average daily dose of warfarin prescribed. All the analyses were performed using the Statistical Package for Social Science (SPSS 10.0 for Macintosh). A two-tailed level of significance of 5% was used for all tests.

Results

Patients' 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. Thus, 147 patients (median age: 42.0 years, range: 15-84) were analyzed. Male sex, indication for anticoagulation, 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*).

Genotypes

Twenty-five patients (17.0%; 95% CI: 10.9-23.1) carried the A20210 allele, whereas 15 individuals (14 heterozygotes and 1 homozygote: 10.2%; 95% CI:5.3-15.1) carried a methionine at position 165 within the exon 5 of the factor II gene (Table 1). Thirty-seven patients (35 heterozygotes and 2 homozygotes: 25.2%; 95% CI:21.6-28.8) showed the A-402 allele and 49 (44 heterozygotes and 5 homozygotes: 33.3%; 95% CI:29.4-37.2) the T-401 allele in the factor VII promoter (Table 1). With regards to the *CYP2C9* (Table 1), 49 patients (33.3%; 95% CI:29.4-37.2), all heterozygotes, carried a cysteine at position 144 within exon 3, and an isoleucine at position 359 within exon 7 (*CYP2C9*2* haplotype). Twenty-six patients (18.7%; 95% CI: 15.5-21.9), 24 heterozygotes and 2 homozygotes, showed an arginine at position 144 within exon 3 and a leucine at position 359 within exon 7 (*CYP2C9*3* haplotype). Two patients carried both a cysteine at position 144 within exon 3 and a leucine at position 359 within exon 7 (*CYP2C9*2+CYP2C9*3* haplotype).

Phenotype analysis

The main characteristics of the groups with different genotypes are shown in the Table 2. Male sex, and averages of the regular estimations of the INR were not significantly different among groups with different factor II (G20210A, Thr165Met) or factor VII (G-402A, G-401T) genotypes or *CYP2C9* haplotypes. Individuals carrying the factor VII GG-402 genotype had more frequently been prescribed other drugs (p=0.05; two-sided Fisher's exact test). Patients carrying the factor VII T-401 allele differed from those carrying the wild genotype for the mean duration of the oral anticoagulation, and number of visits (p<0.05; Mann-Whitney U-test).

The average dose of warfarin received was higher among patients with the factor VII GG-401 genotype

Table 2. Clinical characteristics of the patients divided according to genotype.

	Sex (m/f)	*Mean time on OAT years (SD)*	*Warfarin mean daily dose mg (SD)*	*Mean INR (SD)*	*Patients taking other drugs% (n)*	*Visits n (SD)*
Factor II						
Thr/Thr (n=132) 89.8%	72/60	1.6 (1.4)	5.7 (2.8)	2.55 (0.40)	35.2 (51)	38.6 (23.8)
Thr/Met + Met/Met (n=15) 10.2%	8/7	1.7 (1.4)	4.5 (1.5)	2.46 (0.26)	37.1 (5)	33.3 (21.3)
GG20210 (n=122) 83.0%	69/53	1.7 (2.2)	5.6 (2.8)	2.54 (0.40)	35.2 (79)	27.7 (24.6)
GA20210 (n=25) 17.0%	11/14	1.6 (1.2)	5.1 (2.2)	2.50 (0.33)	52 (12)	29.8 (17.4)
Factor VII						
GG-402 (n=110) 74.8%	59/51	1.7 (2.2)	5.5 (2.9)	2.55 (0.40)	42.7 (47)	27.8 (23.6)
GA-402 + AA-402 (n=37) 25.2%	21/16	1.6 (1.4)	5.8 (2.3)	2.50 (0.36)	24.3 (9)§	28.7 (23.4)
GG-401 (n=98) 66.7%	51/47	1.5 (2.3)	5.9 (2.8)	2.50 (0.40)	36.6 (36)	24.4 (20.4)
GT-401 + TT-401 (n=49) 33.3%	29/20	2.0 (1.6)#	4.9 (2.4)#	2.61 (0.36)	40.8 (20)	35.1 (27.5)#
CYP2C9						
Allele*1 (n=74) 50.3%	44/30	1.8 (2.5)	6.7 (2.9)	2.47 (0.36)	37.8 (28)	30.2 (23.3)
Allele*2 (n=47) 32.0%	25/22	1.7 (1.5)	4.9 (1.8)°	2.58 (0.41)	37.2 (17)	29.1 (23.4)
Allele*3 (n=24) 16.3%	11/13	1.2 (1.3)	3.6 (2.0)°	2.62 (0.43)	37.5 (9)	26.5 (24.3)
Allele*2 + Allele*3 (n=2) 1.4%	0/2	2.3 (2.7)	1.8 (0.1)°	3.00 (0.21)	100.0 (2)	55.0 (58.0)

*OAT: oral anticoagulant therapy. §p: 0.05 vs. GG-402 carriers (two-sided Fisher's exact test). #p<0.05 vs. GG-401 carriers (Mann-Whitney U-test). °p<0.05 vs. CYP2C9*1 carriers (Scheffé's test).*

(5.9 mg) than among patients carrying the T allele (4.9 mg; p=0.017; Mann-Whitney U-test). Anticoagulated patients carrying the *CYP2C9*1* haplotype had been prescribed a higher mean dose (6.7 mg) than patients with the *CYP2C9*2* (4.9 mg; p<0.05; Scheffé's test) or the *CYP2C9*3* haplotype (3.6 mg; p<0.05; Scheffé's test). The dose was even lower in the 2 patients with the *CYP2C9*2+CYP2C9*3* haplotype (1.8 mg) (Table 2). Finally, patients with the factor II Thr/Thr165 genotype had received a slightly higher mean dose of warfarin (5.7 mg) than had patients with the Met165 allele (4.5; p=0.077; Mann-Whitney U-test).

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 was started, sex, average INR, time (in years) on oral anticoagulation, other drugs prescribed, and number of visits. A stepwise analysis revealed the independent nature of the factor II Thr165Met (r^2: 0.034; p=0.009) and of the factor VIIG-401T (r2: 0.021; p=0.037) polymorphisms 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: 0.206; p<0.001). In addition, age when oral anticoagulation was started also had a significant effect (r^2: 0.086; p<0.001).

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 factor II Thr165Met polymorphism (F:4.271; p=0.041), the factor VII G-401T (F:4.862; p=0.029), and the *CYP2C9* haplotype (F:11.134; p<0.001). The adjusted dose of warfarin required was significantly higher in patients carrying the factor II Thr/Thr165, the factor VII GG-401, or the *CYP2C9*1* genotype (Table 3). No significant interaction was found between any of the variables analyzed (*data not shown*).

Table 3. Adjusted mean doses of warfarin prescribed to patients divided according to different genotypes/haplotypes.

	Adjusted mean (mg)	*95% CI*	*SE*	*Significance*
Factor II Thr165Met				
Thr/Thr	4.2	3.1-5.4	0.6	Ref.
Thr/Met+ Met/Met	2.9	1.3-4.5	0.8	0.041
Factor VII G-401T				
GG-401	4.1	2.8-5.3	0.6	Ref.
GT-401 + TT-401	3.1	1.7-4.4	0.7	0.029
CYP2C9				
CYP2C9*1	5.4	4.5-6.3	0.5	Ref.
CYP2C9*2	3.7	2.6-4.7	0.6	<0.001
CYP2C9*3	2.4	1.2-3.7	0.7	<0.001
				0.035*
CYP2C9*2 + CYP2C9*3	2.7	-0.8-6.2	1.8	>0.05

C.I.: confidence intervals. S.E.: standard error. Ref.: reference group.
**vs. CYP2C9*2 haplotype.*

Effect of the factor II Thr165Met polymorphism on prothrombin plasma levels

The possibility of a modulating effect of the factor II Thr165Met polymorphism on prothrombin plasma levels was evaluated in a group of 101 apparently healthy subjects (54 men and 47 women) randomly selected from a Southern Italian population. Genotype frequencies in the sample were 80.2% for Thr/Thr; (n=81), 18.8% for Thr/Met; (n=18), and 2.0% for Met/Met; (n=2). Mean (SD) prothrombin activity of subjects carrying the factor II Thr/Thr 165 genotype was 113.1% (22.6). It was similar to that measured in subjects with the Met165 allele (108.9% [25.5]; Mann-Whitney U-test: p=n.s.). These results did not materially change after having excluded individuals (n=3) carrying the A20210 allele (*data not shown*).

Discussion

Oral anticoagulant therapy is widely used for the management and prevention of venous and arterial thrombosis. The use of the same fixed dose of warfarin for all patients is impractical because patients respond very differently to warfarin. For this reason, the anticoagulant effect of warfarin is measured by a standardized prothrombin time and the dose adjusted accordingly.[29] Variants of the *CYP2C9* locus, which codes for an enzyme that metabolizes warfarin, have been associated with large inter-individual differences in the anticoagulant response to warfarin.[18-26] However, even taking into account *CYP2C9* haplotypes there is still a wide variability in response among subjects receiving warfarin suggesting that additional factors contribute to this variability. Very recently, genetic variations within the genes for vitamin K-dependent clotting factors, namely factor II and factor VII, have been found to predict sensitivity to warfarin therapy.[27] In a cohort of patients followed up from the start of anticoagulation in one clinic, which is part of and fulfils the instructions of the Italian Federation of Anticoagulation Clinics, we investigated whether common variants of the factor II and VII genes contribute significantly to the inter-individual variability in response to warfarin therapy. Carriers of the factor VII GG-401 genotype had received significantly higher doses of warfarin than had carriers of the T allele. The groups of 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 factor VII G-402A polymorphism was not associated with differences in the average dose of warfarin prescribed. As regards factor II, the G20210A polymorphism in the 3' untranslated region did not affect the warfarin dosage, whereas there was trend toward significance for the non-synonymous Thr165Met polymorphism (p=0.077). As expected, carriers of the *CYP2C9*2* and *CYP2C9*3* haplotypes had received significantly lower mean doses of warfarin than had carriers of the *CYP2C9*1* haplotype. The groups of 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, such as the interference of dietary habits or the contemporary prescription of drugs, may affect inter-individual 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.[30] To adjust for these confounding variables, average INR, the mean of all INR results recorded when a patient attended a prescheduled clinic visit, and the contemporary prescription of additional drugs known to be metabolized by the *CYP2C9* cytochrome were also considered in the multivariate analyses. Both the two statistical models applied, multiple linear regression and general factorial ANOVA, confirmed that the factor VII G-401T polymorphism and *CYP2C9* haplotypes significantly influenced the different average doses of warfarin prescribed to attain the prescheduled anticoagulation target. The factor II Thr165Met polymorphism was also significantly associated with the average dose of warfarin prescribed. No relationship was found with other polymorphisms

investigated. All together, the genetic variants investigated accounted for about one quarter (r^2: 0.261) of the inter-individual variability calculated in the present setting.

The biological mechanism by which variants in the *CYP2C9* gene are associated with large inter-individual pharmacokinetic and pharmacodynamic differences in the outcome of warfarin therapy is well-known, individuals with *CYP2C9* gene variants showing a reduced metabolic capacity.[31] Possible explanations for the significant associations of factor II Thr165Met and factor VII G-401T found in the present study are matter of hypotheses. We did not find any association between the factor II Thr165Met polymorphism and prothrombin plasma levels. Thus, we can exclude the possibility that different circulating levels of prothrombin are related to different sensitivities to warfarin therapy. The lack of association with the G20210A polymorphism, which is associated with different plasma levels of prothrombin,[28] adds further strength to this conclusion. The threonine at the residue 165 is conserved in humans, cows and mice while a strictly related amino acid, serine, is found in rats. The Thr165 is close to highly conserved residues, Gly-Pro-Trp-Cis-Tyr-Thr-Thr, which encompass a beta strand. Thus, the substitution of a polar, tiny amino acid, threonine, with a non-polar one, methionine, may induce conformational changes in the three-dimensional structure and this may interfere with the affinity for the γ-glutamyl-carboxylase.

The factor VII G-401T polymorphism is in linkage disequilibrium with other polymorphisms in the promoter region, -323(10/0) and C-122T, and all of them significantly affect plasma levels of factor VII, the T-401 allele being associated with reduced levels.[32] In the present setting, patients with the T allele had been prescribed lower average doses of warfarin, suggesting that different plasma FVII concentrations may influence warfarin sensitivity. At variance with this, the factor VII G-402A polymorphism was not associated with mean doses of warfarin prescribed. The rare A-402 allele is associated with higher levels of factor VII.[33] The two patients carrying the AA-402 genotype were prescribed a higher mean dose (6.9 mg) of warfarin than that prescribed to patients with the GG-402 genotype (5.5 mg) and to the heterozygotes (5.7 mg). However, these differences were not statistically significant. Thus, we cannot exclude other possible explanations for the findings in the present cohort; for example, it is conceivable that the factor VII G-401T polymorphism, or the factor II Thr165Met gene variation is in linkage disequilibrium with unknown allelic variants that modulate sensitivity to warfarin therapy.

The association of polymorphisms in the factor II and VII genes with warfarin sensitivity was previously investigated in a Japanese population.[27] We confirm the association with the factor II Thr165Met polymorphism but, at variance with that study, we did not find a relationship with the factor VII G-402A polymorphism, whereas the opposite was found with the G-401T polymorphism. Although inconsistencies may reflect the play of chance, alternative explanations must be considered. Differences in genetic backgrounds, foremost, and then differences in study design, in the criteria used to select the groups, and in the statistical power of the studies may well account for discrepancies.

In conclusion, we confirm that polymorphisms in factor II and factor VII genes may play a significant role in modulating the anticoagulant effect of warfarin. Whether screening for these polymorphisms would identify patients needing different intensity regimens of anticoagulation is beyond the aims of this study but deserves to be addressed.

RLD'A, GD'A, FC, MC, PDP, VB, EG, MM: gave substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. The authors reported no potential conflicts of interest.

Manuscript received July 14, 2004. Accepted September 10, 2004.

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