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Impact of Genetic Factors (*CYP2C9*, *VKORC1* and *CYP4F2*) on Warfarin Dose Requirement in the Turkish Population

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Abstract: Warfarin has a narrow therapeutic index and displays marked person-to-person variation in dose requirement. Functional polymorphisms at candidate genes can therefore offer utility as biomarkers to individualize warfarin treatment. The main objective of this study was to determine whether and to what extent variability in warfarin dose requirements was determined by polymorphisms in *CYP2C9*, *VKORC1*, *CYP4F2* (rs2108622) and *EPHX1* (rs2292566) in the Turkish population. Patients (n = 107) who had stable doses and international normalized ratio (INRs) at their last three consecutive visits were registered. Their demographic factors, concurrent medications, warfarin-related bleedings or thromboembolisms, smoking, alcohol intake and weekly green vegetable consumption were recorded. From a blood sample, DNA was isolated and genotyped by real-time PCR for polymorphisms of *CYP2C9*, *VKORC1*, *CYP4F2* and *EPHX1*. A regression analysis was used to determine the independent effects of genetic and non-genetic factors on warfarin dose optimization. In our study, in addition to age, genetic variants of *CYP2C9*, *VKORC1* and *CYP4F2* were found to be significant predictor variables for the maintenance dose for warfarin, explaining 39.3% of dose variability. *VKORC1* and *CYP2C9* genotypes remain predictor variables of the warfarin dose, and we first found that *CYP4F2* (rs2108622) contributes to dose variability in the Turkish population as well. These observations may be of benefit to future translation research with a view to global personalized medicine in regions hitherto understudied such as the Turkish population so as to rationalize initial warfarin dose and reduce the burden of frequent INR measurements.

Warfarin is the most commonly used oral anticoagulant for the prevention of stroke in patients with atrial fibrillation, for prophylaxis of venous thromboembolism and pulmonary embolism in patients with prosthetic heart valves and myocardial infarction and for prevention of pulmonary embolism or deep venous thrombosis in patients undergoing orthopaedic surgery or with a history of venous or arterial thromboembolism [1–5]. It is a racemic mixture composed of equal amounts of two enantiomorphs. The levorotatory S-warfarin is four times more potent than the dextrorotatory R-warfarin [6].

S-warfarin is primarily metabolized by CYP2C9. The possession of *CYP2C9*2* or *CYP2C9*3* variant alleles results in decreased enzyme activity and is associated with a significant decrease in the mean warfarin dose because of impaired warfarin metabolism and a higher risk of haemorrhage [7–9]. Although *CYP2C9* polymorphism affects the mean warfarin dose during warfarin dose adjustment, there are still marked remaining individual differences even in *CYP2C9* wild-types in the required dose titration. The target molecule of warfarin, vitamin K epoxide reductase and its gene *VKORC1* was sequenced in 2004 [10,11]. Rieder *et al.* [12] demonstrated that polymorphism in *VKORC1* haplotype groups named as A and B explains approximately 25% of the variance in dose. These two genotypes have also previously been investigated in Turkish patients, and a significant contribution to warfarin dose adjustment was identified [13,14]. After receiving reliable results for *CYP2C9* and *VKORC1* polymorphism relationship with warfarin dose, several new candidate genes have been investigated [15–17]. In addition to genetic factors, several non-genetic factors including age, demographic–dietary factors and concurrent medications were found to have an influence on daily warfarin dose adjustment [1].

Among recently studied candidate genes whose significant contribution to daily warfarin dose has been shown, we selected the *CYP4F2* (rs2108622) and *EPHX1* (rs2292566) gene polymorphisms to achieve an explanation for the interindividual warfarin dose variability in the Turkish population [18–20]. Besides these genetic factors, non-genetic factors including smoking, alcohol intake and vegetable–tea consumption were investigated. In summary, the objective of our study was to determine whether any of these two candidate single-nucleotide polymorphisms (*CYP4F2* (rs2108622)-*EPHX1* (rs2292566)) in addition to *CYP2C9* and *VKORC1* have a relationship with daily warfarin dose amount in Turkish patients, as well as non-genetic factors.

Materials and Methods

Study population. The study protocol was approved by Yeditepe University Clinical Ethics Committee. Patients enrolled at Kartal Koşuyolu Education and Research Hospital Department of Cardiology and Cardiovascular Surgery who had been prescribed warfarin for at least 4 months were selected. Patients were included if they had been on therapy >4 months and their last three international normalized ratio (INR) measurements were within therapeutic range for the same mean daily dose. All individuals

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participating in the study were Turkish. Data were collected on age, body-weight, height, gender, smoking, alcohol intake, number of meals at which vegetables were consumed, grapefruit juice consumption, indication for warfarin use, weekly prescribed warfarin dose, previous history of haemorrhage/embolisms, other medical conditions (if any) and concomitant medications. Blood samples were collected from 263 patients who accepted to participate in the study. Patients who voluntarily agreed to participate in the study signed the appropriate informed consent forms in accordance with the Declaration of Helsinki.

The exclusion criteria included hepatic dysfunction, cancer, advanced heart failure, liver disease and diseases with bleeding tendency. Patients who were receiving any of the following medications that could potentially interact with warfarin were also excluded from the study: antiepileptics including phenytoin and carbamazepine; antituberculosis medications including rifampin (international non-proprietary name INN, rifampicin) and isoniazid; antibiotics including quinolones, tetracyclines, erythromycin, cephalosporins, metronidazole and cotrimoxazole; antifungals including fluconazole and itraconazole; lipid-lowering agents including clofibrate and cholestyramine (INN, colestiramine); anti-arrhythmics including quinidine and amiodarone; histamine-2 blockers including cimetidine and ranitidine; non-steroidal anti-inflammatory agents; allopurinol; cyclosporine (INN, ciclosporin); barbiturates; and oral contraceptives. Additionally, patients hospitalized within the last 4 weeks were excluded from the study group.

In the follow-up period, 156 patients were excluded from the study because of patient incompatibility, comorbidity and concomitant medication. Finally, 107 stable patients were enrolled in the study.

Genotyping. Blood samples (2 mL) were taken from each patient to EDTA-containing tubes, and genomic DNA was isolated from whole blood using MagNA Pure Compact Nucleic Acid Isolation Kit I with MagNA Pure Compact Instrument (Roche Diagnostics, Mannheim, Germany). A real-time PCR device Light Cycler 2.0 (Roche) was used for the detection of polymorphisms in the study. Genotyping of *CYP2C9*1-*2* and *VKORC1* G1639A and C1173T was achieved by *CYP2C9*2*3* Detection Kit (TIB MOLBIOL) and *VKORC1* Detection Kit (TIB MOLBIOL), respectively. Because no commercial kits exist for *CYP4F2* (rs2108622) and *EPHX1* (rs2292566), probes and primers were designed and PCR conditions were verified by TIB MOLBIOL (LightSNiP rs2108622 CYP4F2 and LightSNiP rs2292566 EPHX1 Detection Kit).

Statistical analysis. For the genetic variables, we coded CYP2C9 SNPs as 0 (wild-type), 1 (heterozygous for CYP2C9*2 or CYP2C9*3) or 2 (homozygous for CYP2C9*2 or CYP2C9*3 or compound heterozygous for CYP2C9*2 and CYP2C9*3). Similarly, all other genotypes for VKORC1, CYP4F2 and EPHX1 were coded as 0 (wild-type), 1 (heterozygous) or 2 (homozygous). The daily maintenance dose of warfarin in these different genotype groups was evaluated by ANOVA and used to determine the relationship between genotypes and warfarin amount, and Bonferroni correction was undertaken for precise multiple testing.

For non-genetic factors, associations between warfarin dose and age, height, weight, number of cigarettes (if any), daily consumed tea and green vegetable consumption amount in a week were analysed using Pearson correlation test, and *t*-test was used for evaluation of demographic factors (sex) and alcohol consumption.

Finally, the effects of genetic and non-genetic parameters on daily warfarin dose were assessed by analysing covariables with *p*-values < 0.05 in the multiple regression model. For determination of the deviation from Hardy–Weinberg equilibrium, chi-square test was performed, and then, *p*-value was calculated at 1 degree of freedom. Computations were performed using the SPSS 19.0 statistical programme (SPSS, Chicago, IL, USA).

Results

Between April 2009 and January 2010, 263 patients were enrolled. According to the exclusion criteria defined above, 156 of these patients were excluded. Then, genomic DNAs were isolated from blood samples of the remaining 107 stable patients (54 females and 53 males; mean age 53.89 ± 13.55) whose last three INR values were between the ranges of 2.0 and 3.0. The mean daily warfarin dose for these patients was 5.16 ± 1.95 mg (range 1.43–10.00 mg).

In the study, five SNPs were tested: *VKORC1* (rs9923231, −1639G>A), *CYP2C9* (rs1799853 for *2 and rs1057910 for *3), *CYP4F2* (rs2108622) and *EPHX1* (rs2292566). No significant deviation from Hardy–Weinberg equilibrium was observed for any polymorphism ($p > 0.05$). Differences in the daily maintenance dose of warfarin in the different genotype groups were calculated using ANOVA. Among these genotypes, all SNPs caused a significant difference in the daily warfarin dose amount except from the polymorphism at *EPHX1* (rs2292566).

The relationship between these genotypes and warfarin dose is summarized in table 1. As previously reported, *CYP2C9*2/*3* and *VKORC1* −1639G>A were significantly associated with stable warfarin dose and patients carrying two variant alleles required significantly lower doses ($p = 0.002$ for

Table 1.
Relationships between genotype and warfarin maintenance dose in the entire cohort (n = 107).

	Number of patients (%)	Daily warfarin maintenance dose (mg), (mean ± S.D.) (range)
CYP2C9 genotype		
*1/*1	70	5.43 ± 2.01 (1.50–10.00)
*1/*2–*1/*3	28	5.17 ± 1.63 (2.32–9.64)
*2/*2, *2/*3 *3/*3	9	3.05 ± 1.07 (1.43–5.00)
p-value (ANOVA)		0.002 (Between groups)
		0.010 (*1/*1–*1/*2.3)
		0.001 (*1/*1–*2.3/2.3)
VKORC1 genotype		
GG	27	6.00 ± 1.91 (3.57–9.64)
GA	55	5.37 ± 1.86 (1.43–10.00)
AA	25	3.79 ± 1.49 (1.50–6.43)
p-value (ANOVA)		<0.001 (Between groups)
		0.001 (GG-GA)
		<0.001 (GG-AA)
CYP4F2 (*rs2108622*) genotype		
CC	40	4.53 ± 1.73 (1.96–7.50)
CT	49	5.58 ± 2.24 (1.43–10.00)
TT	18	5.42 ± 1.10 (3.21–7.14)
p-value (ANOVA)		0.032 (Between groups)
		0.030 (CC-CT)
EPHXl (*rs2292566*) genotype		
GG	77	5.26 ± 2.24 (1.43-10.00)
GA	25	4.92 ± 1.47 (2.50-7.50)
AA	5	7.07 ± 2.21 (4.29-9.29)
p-value (ANOVA)		0.728 (Between groups)
Total	107	5.16 ± 1.95 (1.43–10.00)

CYP2C9 and $p < 0.001$ for *VKORC1*, respectively). In addition, variance at *CYP4F2* (rs2108622), which has never been mentioned before in the literature as affecting the warfarin maintenance dose in Turkish patients, was found to have significant influence. Patients with the wild-type CYP4F2 (CC) sequence required a mean dose of 4.53 ± 1.73, while individuals with one or two variant alleles were found to require higher doses [5.58 ± 2.24 for CT ($p = 0.032$) and 5.42 ± 1.10 for TT, respectively]. Variance in *EPHX1* (rs2292566) showed significant contribution to warfarin dose adjustment in previous studies, whereas no such effect was observed in our study.

Figure 1 shows the cumulative effect of the variant alleles of *CYP2C9**2/*3, *VKORC1* −1639G>A and *CYP4F2* (rs2108622) on the maintenance dose.

Besides genetic factors, eight non-genetic factors of 107 patients were also reported and the relationships between these variables and warfarin maintenance dose were analysed (table 2). Among these characteristics, variations in age showed a significant effect on the maintenance dose with a *p*-value of 0.024.

Covariables with *p*-values < 0.05 by statistical analysis were entered into a regression analysis, and *CYP2C9*, *VKORC1* and *CYP4F2* (rs2108622) genotypes, as well as age, explained 39.3% of the overall interindividual differences in the warfarin dose, with *CYP2C9* accounting for 19%, *VKORC1* for 14.7%, *CYP4F2* (rs2108622) for 2.8% and age for 2.8% (table 3).

Discussion

To our knowledge, this is the first study to show the influence of *CYP4F2* (rs2108622) variance on warfarin maintenance dose in Turkish patients. The regression model included age and three genotype variants, namely *CYP2C9*, *VKORC1, CYP4F2* (rs2108622), and these variants accounted for 39.3% of the dose variability.

In recent studies, *EPHX1*, encoding microsomal epoxide hydrolase, has been suggested as a new genetic variant affecting the warfarin maintenance dose significantly [21–24]. *EPHX1* rs2292566 was selected as a synonymous polymorphism candidate to investigate the dose variations further

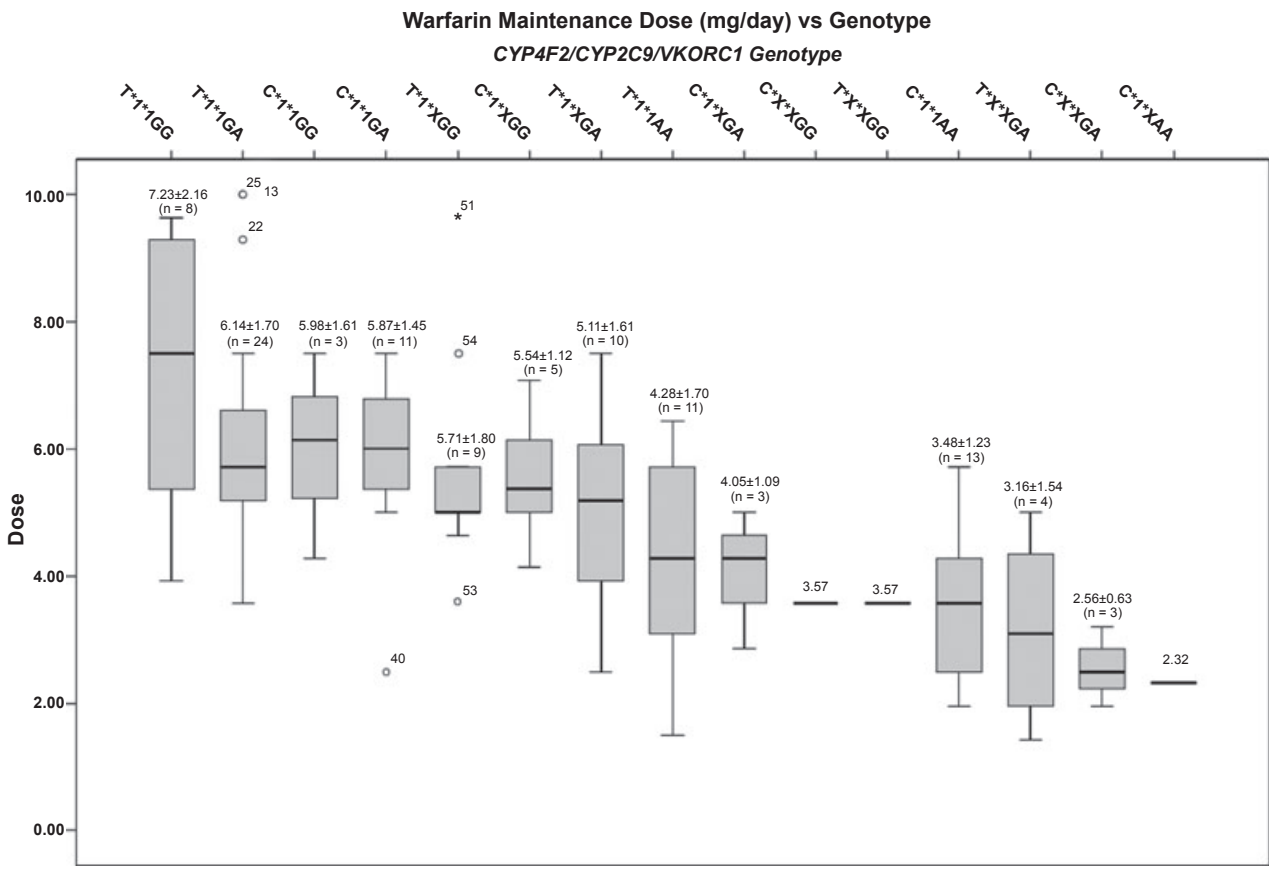

Fig. 1. Daily warfarin maintenance dose requirements and *CYP2C9/VKORC1/CYP4F2* genotypes. Variant alleles for *CYP4F2* are shown as C (wild-type) and T [heterozygous (CT) or homozygous (TT)]. CT and TT genotypes were grouped together due to their similar daily warfarin dose requirements. *CYP2C9* and *VKORC1* genotypes were quoted as *1-*1 (wild-type), *1/*X (carrying one variant allele),*X/*X (carrying two variant alleles) and GG (wild-type), GA (heterozygous), AA (homozygous); respectively. Below the box plots, mean value of the daily warfarin maintenance doses and sample numbers are shown with respect to different genotypes. The circles and the asterisks show outliers and far outliers, respectively, and the numbers adjacent to these define their order in data.

Table 2.
Demographic, biologic, clinical and therapeutic characteristics of the 107 patients in whom warfarin therapy was initiated and the relationship of these characteristics to the maintenance dose.

	N (%) or mean ± S.D. (range)	*p*-value (test)
Age (years)	53.89 ± 13.55 (25–79)	0.024 (Pearson correlation)
Female patients/ male patients	54/53	0.104 (Two independent *t*-test)
Height	164.99 ± 8.18 (150–189)	0.928 (Pearson correlation)
Weight	76 ± 11.98 (54–115)	0.288 (Pearson correlation)
Tea	4.93 ± 4.90 (0–30)	0.350 (Pearson correlation)
Smoking	0.78 ± 2.94 (0–20)	0.320 (Pearson correlation)
Vegetable consumption	68/35/4	0.780 (Pearson correlation)
Alcohol	7	0.870 (Two independent *t*-test)
Bleeding events	38	0.092 (two-sided Chi-square test)
Thrombosis	5	NT
Indication of warfarin therapy		
Heart valve prosthesis	81 (76)	
Venous thromboembolism	13 (12)	
Atrial fibrillation	8 (7)	
Cardiac surgery	5 (5)	
Stable chronic medical conditions		
Hypertension	12	
Chronic heart failure	2	
Diabetes mellitus	9	
Rheumatic disease	5	
Hypothyroidism	1	
Gastrointestinal disorders	7	
Respiratory insufficient	2	
Renal impairments	4	

among Turkish patients, with an assumption of the haplotype resemblance between Turks and Caucasians [24]. Although genotype frequency values were close to the study conducted among Caucasian patients, in contrast to the previous study, no significant relationship was observed between dose variations and allele types for Turkish patients**.** The significance value of the correlation test was much higher for our study population ($p = 0.728$ *versus* $p = 0.043$), and we speculated that the age differences of the study populations were the main reason. The mean age value in that study was 86.2 years, whereas in our study, it was 53.89 years. Sequencing the *EPHX1* gene in Turkish patients or investigating other SNPs in *EPHX1,* which were associated with warfarin dose, might be subjects for further investigations.

Patients with the *CYP4F2* rs2108622 polymorphism are likely to have elevated hepatic levels of VK1, necessitating a higher warfarin dose to elicit the same anticoagulant response [15]. Several studies performed in recent years have demonstrated that rs2108622 polymorphism explains up to 5% of the

Table 3.
Characteristics of the final forward stepwise regression model for fitting the warfarin maintenance dose (n = 107).

	Variable	Parameter estimate	Partial R^2	*p*
0	Intercept	8.308		<0.001
1	*VKORC1* −1639A, per A allele	−1.499	0.147	<0.001
2	*CYP2C9* *2 or *3, per variant allele	−1.33	0.19	<0.001
3	Age (per year)	−0.028	0.028	0.013
4	*CYP4F2*, per T allele	0.505	0.028	0.017

The daily warfarin dose (in mg) predicted by the model can be obtained as follows: 8.308 − (1.499 × number of variant *VKORC1* alleles) − (1.330 × number of variant *CYP2C9* alleles) − (0.028 × age (years) + (0.505 × number of variant *CYP4F2* alleles) + e.

variances of dose among the warfarin patients, which was 2.8% in this study [25]. Allele frequencies for wild-, hetero- and mutant-type patients were found to be 37%, 46% and 17%, respectively. These allele frequencies show resemblance with Middle East population wild-, hetero- and mutant types with 36%, 48% and 16%, respectively [26]. Patients carrying *CYP4F2* (rs2108622) allele were found to require a higher daily warfarin dose for heterozygote and mutant individuals of 23.18% and 19.65%, respectively, than wild-types.

In this study, observed allele frequencies for *VKORC1* wild-, hetero- and mutant individuals were 25%, 51% and 24%, respectively, which is compatible with two previously conducted Turkish studies with values of 28.8% and 36% for wild-types, 42.4% and 48% for heterozygotes and 28.8% and 16% for mutant types. We determined a reduction in warfarin dose requirements for heterozygote and mutant genotypes of 10.50% and 36.83%, respectively, compared with the wild genotype. These values reached to significance for the mutant patients ($p < 0.001$), meaning that sensitivity to the drug is highest for this group of patients, and lower dose regimens should be applied. Approximately 15% of overall interindividual dose variability with *VKORC1* variants could be explained; however, it was lower than reported in other Turkish and international studies (20–35%).

In our study, individuals' heterozygous for the *CYP2C9* *2 or *3 allele required 4.79% lower daily maintenance dose than homozygous wild-type individuals. Additionally, individuals carrying more than one variant allele required a 43.83% lower warfarin dose with respect to wild-type patients ($p < 0.05$). These results were able to explain 19% of the dose variability. In two previous studies focusing on Turkish patients, *CYP2C9* polymorphism accounted for 8% and 13% of dose variability [13,14]. Our results are in concordance with the previous ones and confirm the well-known association between *CYP2C9* and daily warfarin dose.

Besides genetic factors, age was also included in the regression model as a non-genetic factor influencing the daily dose adjustment of warfarin. Dose requirements decrease with age by 8–10% per decade of life, owing to reduced clearance and/or increased responsiveness [27,28]. A study of 297

patients on stable warfarin doses reported that mean warfarin daily dose requirements fell by 0.5–0.7 mg per decade between the ages of 20 and 90 years [29]. Multiple linear regression models used to develop warfarin dosing algorithms have consistently found age to be a significant contributor to variability in dose requirements, and it has been established that age is associated with warfarin maintenance dose ($p < 0.05$), accounting for 2.8% of the dose variability. Although the effect was still significant, it was concluded that the lower mean age compared to previous studies resulted in relatively smaller impact.

There is conflicting evidence on the association between warfarin doses and dietary factors such as alcohol and tea consumption, smoking and vitamin K intake. High intake of fat-soluble vitamin K theoretically diminishes the effect of warfarin, which interferes with coagulation by inhibiting regeneration of the reduced form of vitamin K. In our study, we did not observe a significant relationship between dietary factors (vitamin K intake, alcohol/tea consumption and smoking) and warfarin maintenance dose contrary to the findings in previous studies [30–32]. To investigate the association between dietary factors and warfarin dosage, more controlled studies are needed to achieve reliable results. As other non-genetic factors, height and weight values of individuals were recorded to determine the relationship with warfarin dose adjustment. However, in contrast to previous studies, no significance was reached in our study ($p = 0.93$ and $p = 0.29$, respectively).

Pharmacogenetics, a science in its relative infancy, has the potential to improve the safety and efficacy of medications. Warfarin with its very narrow therapeutic index has been one of the target drugs of pharmacogenetics. To date, a correlation between steady-state warfarin dose and several genetic, non-genetic factors has been demonstrated by numerous previous studies. In our study, *CYP2C9* and *VKORC1* −1639G>A variant genotypes with age remained predictor variables of the warfarin dose and significant determinants of the warfarin response as expected, while the impact of *CYP2C9* was surprisingly higher than *VKORC1,* which was interpreted as the effect of lower mean age. In addition, variance at *CYP4F2* (rs2108622), which was first mentioned in the literature as affecting the warfarin maintenance dose in Turkish patients, was confirmed to have significant influence. With larger studies, more precise results and a more solid knowledge could be gained for warfarin dose adjustment. By taking into account the ethnic differences in the pharmacogenetics of the response to warfarin, the translation of this knowledge into clinical guidelines and designing of algorithms to aid dosing in clinical practice can be useful for determination of initial warfarin dose, reduction in the burden of frequent INR measurements and improvement in the safety by reducing the risk of overanticoagulation in Turkish patients.

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No competing financial interests exist.

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