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The Effect of *CYP2C9* and *VKORC1* Genetic Polymorphism on Warfarin Dose Requirements in a Sample of Iraqi Patients

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Abstract

Background: Warfarin is the most widely used oral anticoagulant for the prevention and treatment of thromboembolic disorders. Because of narrow therapeutic index and various genetic and non-genetic factors that influence the disposition of the drug, its dose undergoes a great variability. The aim of this study was to determine the allelic variants of CYP2C9 and *VKORC1* genes in Iraqi patients, and to investigate the contribution of genetic on warfarin dose requirements.

Methods: A cross sectional study was carried out on a sample of Iraqi patients from Baghdad city who were admitted to Ibn AL-Bitar Specialized Center for cardiac surgery. Blood samples of all patients were collected for both hematological and genetic analysis utilizing standard techniques**.**

Results: The frequency of *CYP2C9*3* allele was 9.4% whereas that of *CYP2C9*2* allele was 13.7%. The frequency of (*VKORC1-1639G*) allele was 58.75% and the frequency of (*VKORC1-1639A*) allele was 41.25%. Patients' daily warfarin doses were administered according to their genotype.

Conclusion: It can be concluded that *CYP2C9*3* and *VKORC1* had significant effect on warfarin dose. New warfarin-dosing algorithm was developed based on *CYP2C9*3* and *VKORC1*genotypes for predicting the required dose of warfarin.

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Introduction

Warfarin is used for the prophylaxis and treatment of venous thrombosis such as pulmonary embolism, deep venous thrombosis, thromboembolic complications and stroke associated with valvular and non valvular fibrillation and cardiac valve replacement (1). The safety of warfarin therapy is

dependent on maintaining the prothrombin time expressed as international normalized ratio (INR), which is a ratio of the time required for the patient's blood to coagulate relative to a standardized coagulation time, within the desired therapeutic range (2). The wide inter- individual variation in drug response, and narrow therapeutic range with the risk of hemorrhage, all make warfarin a challenging drug clinically (3). Warfarin dose varies among individuals and all factors responsible for the variability have not been known. Known factors that affect an individual's response to the dose of warfarin include non-genetic factors (e.g., age, race, body weight, sex, concomitant medications including those that compete for binding to albumin, comorbidities, diet, and nutritional status) and genetic factors CYP2C9 and VKORC1 genotypes (4).

Warfarin consists of two racemic isomers of (R) - and (S) – enantiomers that differ in their plasma concentrations, antithrombotic potency and also in the enzymes responsible for their metabolism. It has been shown that the S-form is 3–5 times more active than the R-form concerning the inhibitory effect on the target, and accounts for 60% to 70% of warfarin's anticoagulant activity. Both enantiomers are metabolized by cytochrome P450's; S-warfarin is metabolized almost exclusively by CYP2C9, whereas CYP1A2, CYP1A1 and CYP3A4 are responsible for the metabolism of the R-enantiomer (5).

Genetic polymorphism of CYP2C9 is responsible for the pharmacokinetic effect on Warfarin metabolism (6) while genetic variations of VKORC1 are responsible for the pharmacodynamic effect on Warfarin (7). Warfarin has become a case study for personalized medicine algorithms incorporating selected SNPs in 2 genes, CYP2C9 and VKORC1, enhanced dose prediction compared with algorithms based on clinical and demographic factors only (8).

The aims of the present study were:

1- Determining the frequency of VKORC1-1639A&VKORC1-1639G and CYP2C9 *1,*2,*3 alleles among Iraqi populations and their relations to Warfarin metabolism in patients under Warfarin therapy.

2- Investigating the contribution of genetic factors on the variability of warfarin dose requirements & optimizing warfarin dose to minimize the adverse effects (haemorrhage or thrombosis).

Materials and Methods

This study was conducted in January 2017, a total of 80 patients (41males and 39 females) aged 30 to 70 years were enrolled. The participants with cardiovascular disease (mainly cardiac valve replacement) were recruited from Ibn AL-Bitar Specialized Center for cardiac surgery in Baghdad.

The inclusion criteria of the study were Iraqi patients who had attended the hospital and received continuous warfarin therapy for ≥ three months due to thromboembolic disorders including deep venous thrombosis, surgical cardiac valve replacement and those at risk of stroke.

The exclusion criteria of the study were elevated prothrombin time (PT), smoking, drinking alcohol, consumption of food and herbs that interact with warfarin and affect PT level, renal failure, hepatic failure, pregnant women, advanced physiological age, terminal illness and medications that affect the pharmacokinetic or dynamic of warfarin (e.g., enzyme inducer or inhibitor). Demographics of gender, age, weight, and height, Body mass index (BMI) as well as date of warfarin initiation and indication were recorded.

The ethical approval (5/1/52/25 at 1/9/2017) of this study was obtained from the Medical College / Al-Nahrain University and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Each patient donated a sample of venous blood using disposable 5 ml Syringe. A volume of 2.5 ml blood was transferred to a tube that contained 0.5 ml sodium citrate. The tubes that contained the blood were mixed gently and immediately transferred to the laboratory for PT&INR measuring. INR was calculated based on PT. For molecular study, the remaining 2.5 ml of the blood was transferred to EDTA-tube and mixed gently before transferring to the laboratory. DNA was extracted using ReliaPrep™ Blood gDNA Miniprep system (promega, USA) following the manufacturer's protocols, then used as the template for PCR amplification. DNA concentration was measured with QuantiFluor dsDNA Dye kit on a Quantus Fluorometer device (promega, USA) designed to provide highly sensitive fluorescent detection when quantifying nucleic acids and proteins. All the primers for PCR amplification and DNA sequencing were designed and synthesized (Macrogen, korea). Primer optimization was done before PCR run, as the primers were designed in order to determine the suitable temperature for the primers to bind with.

Genotyping of CYP2C9

Conventional PCR was done for the detection of *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910) SNPs. PCR was carried out on thermal Cycler (BioRad, USA). DNA samples were amplified by polymerase chain reaction (PCR) in a final volume of a 25 µL each with 12.5 master mix (promega, USA), 1µL of each primer (25 nmol), 2 µL of DNA template and the volume was completed to 25ul using nuclease- free water. Sequences for the forward and reverse primers have been shown in Table 1. The PCR conditions for *CYP2C9* consisted of one cycle of initial denaturation at 94˚C for 5 min. followed by 30 cycle of denaturation at 94 ˚C for 40 sec, annealing at 58 ˚C for 30 sec, and extension at 72 ˚C for 30 sec with the final extension at 72˚C for 10 min. Finally the PCR products were visualized by UV transilluminator on 2% agarose gel stained with ethidium bromide to confirm purity & mobility. The PCR products were sent for Sanger sequencing using AB13730XL, automated DNA sequencer by Macrogen Corporation – South Korea. Data were received after 20 days to 1month. DNA sequences were analyzed using genius software (version R10, USA) in order to detect the genotypes for *CYP2C9*2*, *CYP2C9*3* gene.

Genotyping of VKORC1

The principle of this test depends on two independent PCR reactions for each allele (A or G). The SNPs were genotyped using PCR-CTPP (PCR with confronting two-pair primers). This method requires two independent reaction with two specific primer pairs for each allele specific amplification; F1 and R1 for X allele, F2 and R2 for the Y allele and the end base of R1 and F2 should be the position of SNP. The PCR amplifies three different-sized bands of DNA; between F1 and R1, between F2 and R2, and between F1 and R2 (9, 10). The primer sequences for *VKORC1*G-1639 according to Breslauer *et al.* 1986 (11) have been shown in Table 1. DNA samples were amplified by polymerase chain reaction (PCR) in a final volume of a 20 µL each with 10 master mix (promega, USA), 0.8µL of each primer (30nmol), 2µL of DNA template and the

volume was completed to 20µl using nuclease- free water. The PCR conditions for *VKORC1* gene consisted of one cycle of initial denaturation at 95 ˚C for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min with final extension at 72°C for 5 min. The PCR products were visualized by UV transilluminator on 2% agarose gel stained with ethidium bromide.

Table 1. Primers' sequences for CYP2C9*3, CYP2C9*2 and VKORC1

SNP	Product size (bp)	RS		Primers and sequences
CYP2C9*2 **430C>T**	502	1799853	F	GGAGGATGGAAAACAGAGACTTAC
			R	AAATGAACCTTTTATACCCACACTGT
CYP2C9*3 **1075A>C**	435	1057910	F	ACCTTCATGATTCATATACCCCTGA
			R	TTTATAGCCCCAAACTGGAAACAAG
VKORC1 **-1639A**	119	9923231	F1	CAC AGA CGC CAG AGG AAG AGA G
			R1	CGT GAG CCA CCG CAC CT
VKORC1-1639G	208		F2	GAA GAC CTG AAA AAC AAC CAT TGG CCG
			R2	CTC AGC CTC CCA AGT AGT TTG G

Statistical analysis

All the variables are presented as mean and standard deviation (SD).The daily maintenance dose of warfarin in the different genotype groups was evaluated by t-test and ANOVA. Correlation between warfarin dose and *CYP2C9*2*, *CYP2C9*3* and *VKORC1* were analyzed using Pearson correlation coefficient. The genotype frequencies for each polymorphism were determined. Multiple linear regression was performed to model the relationships of warfarin dose with the variables measured and used to develop a warfarin dosing algorithm. A P-value of ≤0.05 was considered to be statistically significant. All Statistical analyses were performed using SPSS 17.0 software.

Results

Characteristics of the studied patients

A total of 80 patients were enrolled in this study .The demographic and clinical features including age, gender, body mass index (BMI), the underlying diseases, warfarin dose and INR have been summarized in Table 2.

Table 2. Demographic characteristics of the studied patients (n=80)

Variable		Value
Gender	Male	41 (51.25%)
	Female	39 (48.75%)
Age (Years)		47.65 ± 10.12
Weight (kg)		74.33 ± 9.88
Height (cm)		168.68 ± 9.48
Body mass index (kg/m2)		26.09 ± 2.33
Average of INR		2.315 ± 0.823
Warfarin dose (mg/day)		4.29 ± 1.69
Primary reason for anticoagulation	Stroke	6 (7.5%)
	CVR	70 (87.5%)
	DVT	4 (5%)
History of concomitant disease	Hypertension	51 (63.75%)
	Diabetes mellitus	11 (13.75%)
	None	18 (22.5%)

Conventional PCR results for CYP2C9 and VKORC1gene

In the present study, distribution of the variant alleles of *CYP2C9*2, CYP2C9*3 &VKORC1* were analyzed for their prevalence in the population included in the study.

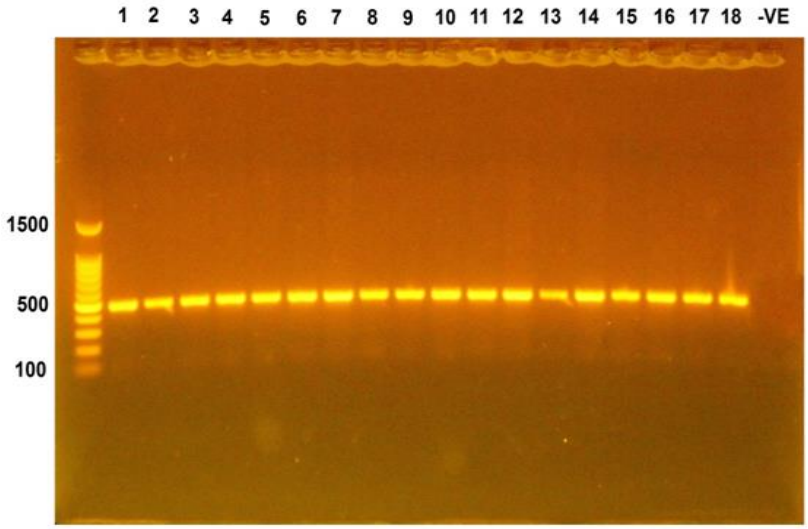

Figure 1. CYP2C9*2 (rs1799853) conventional PCR product (502bp) on gel electrophoresis

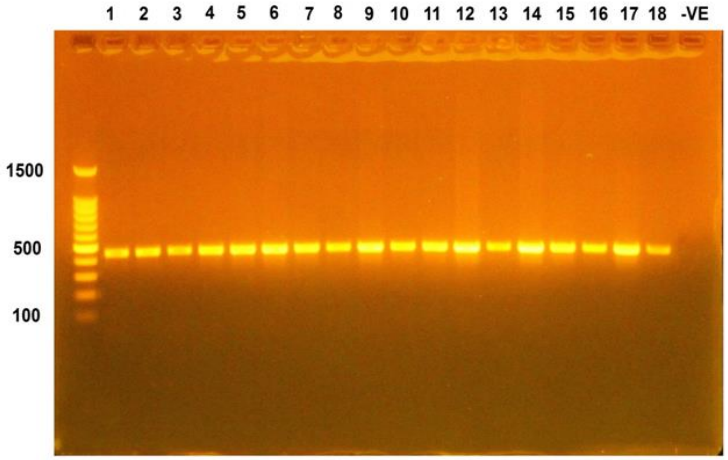

Figure 2. CYP2C9*3(rs105791) PCR products (435pb) on gel electrophoresis

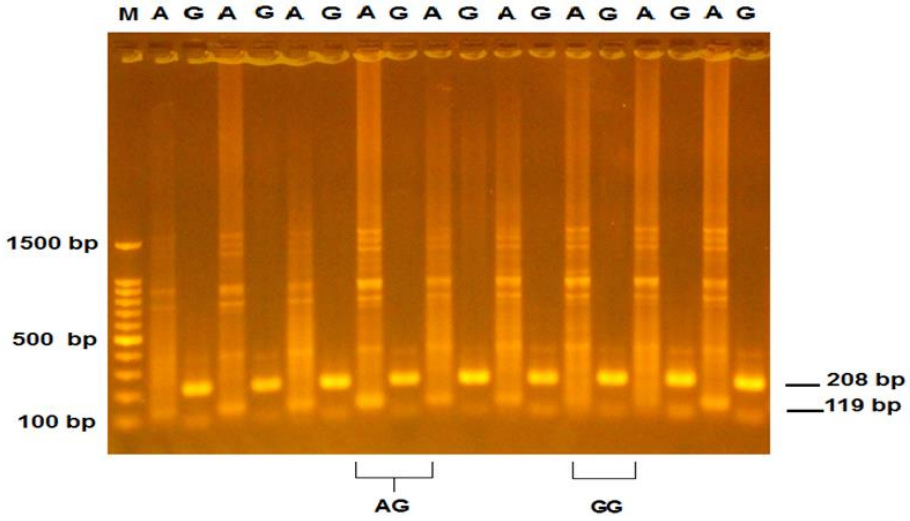

Figure 3. VKORC1 (rs9923231) polymorphism conventional PCR product on gel Lane M, a100-bp ladder, A allele appear at 119bp, G allele appear at 208 bp.

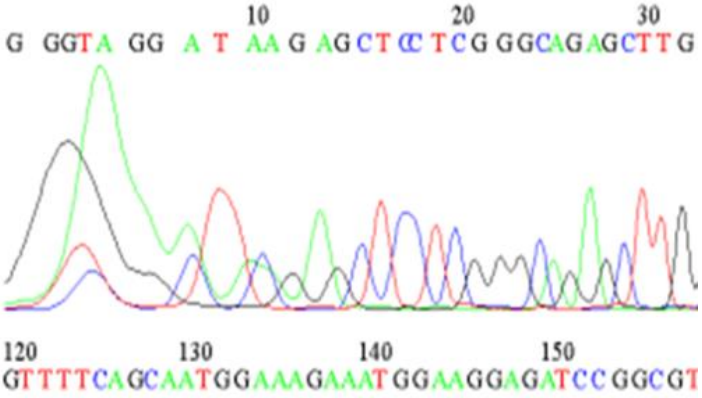

Figure 4. Sample of DNA sequence for CYP2C9*3 gene

Genetic Characteristics of the studied patients

The allelic &genotypic frequencies of the tested SNPs obtained from 80 patients have been shown in Table 3.

Table 3. Genotype of the studied patients with warfarin dose distribution for each genotype

GENE	SNP	Genotype	n	Patients (%)	Allele	Allele%	Warfarin dose (mg) Mean± SD	r	p-value
CYP2C9*2	430C >T	CC	61	76.3	C	138 (86.3%)	4.377 ± 1.71	-0.146	0.195
		CT	16	20	T	22 (13.7%)	4.25 ±1.65		
		TT	3	3.8			2.667 ± 0.577		
CYP2C9*3	1075A>C	AA	66	82.5	A	145 (90.4%)	4.47 ± 1.72	-0.279	0.012
		AC	13	16.3	C	15 (9.4%)	3.576 ± 1.115		
		CC	1	1.3			1		
VKORC1	1639G>A	GG	24	30	G	94 (58.7%)	4.833 ± 2.07	-0.236	0.035
		GA	46	57.5	A	66 (41.2%)	4.15 ± 1.409		
		AA	10	12.5			3.6 ±1.663		

Warfarin dose association with genotype

The mean warfarin dose per day was higher in the patients with the *CYP2C9*2* (CC) genotype (4.377 ± 1.71 mg) than in those with the *CYP2C9*2* (TT) genotype (2.667 ± 0.577mg). The mean warfarin dose per day was higher in the patients with the *CYP2C9*3* (AA) genotype (4.47 ± 1.72 mg) than in those with the *CYP2C9*3* (AC) genotype (3.576 ± 1.115 mg). Patients having *VKORC1* (GG) genotype received higher dose of warfarin per day (4.833 ± 2.07mg) than those with the AA genotype (3.6 ± 1.663 mg) as shown in Table 3.

Correlation of genetic variables with dose

The value of Pearson correlation coefficient revealed that warfarin dose had a significant negative correlation with *CYP2C9*3* and *VKORC1* genes meaning that the presence of *CYP2C9*3* and *VKORC1*genes in patients will decrease the required dose of warfarin while warfarin dose showed non-significant but negative correlation with *CYP2C9*2*gene.

Multiple linear regression equation (Step wise)

The multiple regression model was performed to estimate the relative contributions of genetic and non –genetic factors on warfarin dose. Forward stepwise multiple linear regression was used and the most important variables for determining inter-individual variations of warfarin dose were gender, weight, and *CYP2C9*3, VKORC1* genes that explained 53.8 % of the variance in warfarin dose in Iraqi patients based on genetic polymorphisms and non- genetic factors. The contribution of inter-individual variables to warfarin dose was 29.8% for weight, 40.2 % for gender, 51.2% for *CYP2C9*3* and 53.8 % for *VKORC1* (Table 4).

Table 4. Multiple linear Regression equations for predicting warfarin daily dose

linear Regression equation	P-value	R^2%
dose= -2.658+0.093 (weight)	0.000	29.8
Dose= -7.170+0.128 (weight)+1.286 (gender)	0.000	40.2
Dose= -6.053+0.134 (weight) +1.323 (gender) - 1.332 (CYP2C9*3)	0.000	51.2
Dose= -4.845 + 0.130 (weight) + 1.254 (gender) - 1.337 (CYP2C9*3)- 0.432 (VKORC1)	0.000	53.8

Gender: input 2 for female and 1for male; weight: input weight in kg; VKORC1 genotype: input 1 for GG, 2 for GA, and 3 for AA; CYP2C9*3: input 1 for AA, 2 for AC, 3 for CC

The validation for pharmacogenetic dose algorithm

To validate this pharmacogenetic dose algorithm, the predicted doses were calculated using multiple regression model and based on weight, gender *CYP2C9*3* and *VKORC1*, the calculated doses were compared with the actual doses of warfarin in the same patients. Pearson correlation coefficient showed a very strong and highly significant relationship between the calculated and the actual dose (r=0.733, p=0.000).

Figure 5 demonstrates the calculated and the actual doses of warfarin.

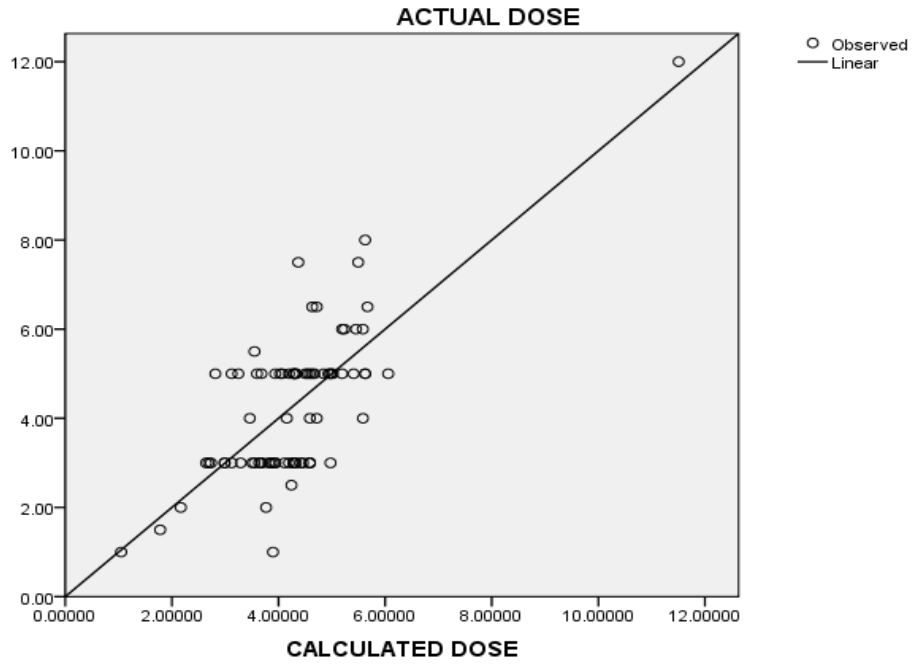

Figure 5. Predicted dose calculated using multiple regression models versus actual dose of warfarin in patients within normal INR range. The actual dose has been represented by circle.

The negative differences between the actual dose and the predicted dose in some cases were because the actual dose is less than the calculated dose, but in most cases the calculated doses were very close to the actual dose and that support its clinical use for treating patients with warfarin.

Discussion

This study was designed to investigate the frequency of *VKORC1 (G-1639A)* & *CYP2C9**1,*2,*3gene polymorphisms in Iraqi patients and the impacts of genetic and non-genetic factors on the required warfarin dose.

The results of the current study confirmed that *VKORC1* (r= - 0.279, p=0.012) *& CYP2C9*3* (r= -0.236, p=0.035) had significant negative correlation with warfarin dose; that is, the presence of the genetic variants will decrease warfarin dose. Warfarin negatively correlated with *CYP2C9*2* gene but this correlation failed to reach the statistical significant level (r= -0.146) in the patients enrolled in this study. D'Andrea *et al*., 2005 (12) suggested that genetic polymorphisms in *VKORC1* may explain a greater proportion of warfarin dosing variability compared with polymorphisms in other genes involved in the biochemical pathway of warfarin. As mentioned before, the main target of warfarin is *VKORC1* and polymorphism of the promoter of this gene (G-1639A) determines the patient's sensitivity to warfarin through lowering the rate of synthesis of this enzyme in patients having the A allele. Patients with the (A/A) genotype require the lowest warfarin doses as compared to heterozygous carriers (G/A) and homozygous carriers (G/G) (2).

In the present study, the mean warfarin dose was higher among patients with the *VKORC1* (-1639) wild types (GG) genotype than in those with heterozygotes genotype (GA) and the homozygous genotype (AA) required the lower dose. Several other studies agree with this finding (2, 13).

In this study, there were significant differences in mean required doses between each of the variant alleles of *CYP2C9* genotypes and the wild type. The highest daily warfarin dose was administered for carriers of the wild type (*1/*1) genotype, while carriers of *CYP2C9*3/*3* were treated with the lowest warfarin doses. Similar results have been reported in previous studies (14, 15). Most of our study population were carriers of the wild type genotype (62.5%) and carriers of *CYP2C9*1/*2* did not differ in warfarin required dose. The *CYP2C9*3* allele was a significant candidate influencing warfarin maintenance doses. There were significant differences in mean warfarin dose between the variant alleles and the wild type (P=0.030).

The *CYP2C9*2* allele was found among Iraqi patients, but there was no significant differences in mean of warfarin required dose between these variant alleles and the wild type (P=0.234).

Results of the present study is extended to demonstrate the impacts of genetic polymorphism of *CYP2C9* and *VKORC1* gene with inter-individual variables including weight and gender in a sample of patients using the multivariate regression mode to account for nearly 54% of the variability in warfarin daily dose requirement. *CYP2C9*3* and *VKORC1* genotypes produced the best model for estimating warfarin dose, having the largest R^2 value of 53.8%.

Dose=-4.845+0.130 (weight) +1.254 (gender)-1.337 (*CYP2C9*3*)-0.432 (*VKORC1*)

Choi *et al*., 2011 (14) used multiple regression model to explain 35% of the variance in warfarin dose in Korean patients based on genetic and non -genetic factors. In Japanese patients, the model using multiple regression analysis including age, sex,

weight and three genetic polymorphisms accounted for 33.3% of total variations in warfarin dose (16). Considering these racial differences in warfarin treatment developing pharmacogenetic dosing algorithm specific for each racial group helps to reduce the adverse effects associated with warfarin use. In the present study, doses estimated by pharmacogenetic algorithm were validated and the calculated doses were compared with the actual doses of warfarin in the same patients and were significantly closer to the required stable therapeutic dose. Pearson correlation coefficient showed significant relationship between the calculated and the actual dose (r=0.733, p=0.000). Significant correlation between calculated and the actual dose have been presented by other studies as well (2, 13-15). This suggests the clinical utility of pharmacogenetic algorithm for treating patients with warfarin. No algorithm that uses genotype to predict a dose on day one has yet been developed for warfarin in Iraqi patients and this algorithm is important in maximizing the benefit of using genotypes to guide anticoagulant dose at the beginning, so that the patient can maintain the target INR for longer period and experiences less complications. This study has demonstrated that the use of pharmacogenetic- based dosing, however there were several limitations. Despite the current knowledge of pharmacogenomics and clinical factors, the source of more than 40% of the variability in warfarin dose has remained unclear. Additional genetic factors, including multidrug resistance1(MDR1), Apo lipoprotein E, and possible genes encoding additional components of the vitamin K epoxide reeducates complex, as well as concomitant medications might be responsible for the observed inter individual variability in warfarin dose requirement (17). Further study is required to expand the sample size. This study explained 53.8% of the variance in warfarin dose in Iraqi patients using a multiple regression model based on genetic polymorphisms of *VKORC1*, *CYP2C9*3* and the non-genetic indicators of gender, weight the current model will continue to evolve after the discovery of additional genes or new contributing factors.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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