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Research Article
Effect of *CYP2C9*2* and *VKORC-1639G/A* Polymorphisms on Warfarin Doses Requirements in Sudanese Patients

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Abstract

Background and Objective: The variation in response to Warfarin is related to clinical and genetic determinants. This study aimed to explore the effect of *CYP2C9*2* and *VKORC-1639G/A* polymorphism on Warfarin dose requirements in Sudanese patients. **Materials and Methods:** This was a descriptive cross-sectional study conducted in the Sudan heart centre, Khartoum-Sudan. A total of 165 Sudanese patients under Warfarin therapy attending the Sudan heart centre during the study period were selected. Citrated anticoagulated whole blood samples were collected from all participants. Plasma was separated for PT-INR measurements, while Buffy coat was used for genomic DNA extraction and subsequent PCR-RFLP analysis for *CYP2C9 430C>T* and *VKORC1-1639G>A* polymorphism. **Results:** The study showed patients that carriers of *CYP2C9 430C>T* or *VKORC1-1639G>A* polymorphisms had significantly lower Warfarin dose requirements compared with wild types. The p = 0.04 and 0.004, respectively. The study also revealed that the combined effects of clinical and genetic factors explained 30.1% of the variation in Warfarin dose. The clinical factors include age, gender, indication for therapy, comorbid disease and concurrent medications. In this study, the *VKORC1-1639G>A* polymorphism was the major predictor of Warfarin dose, explaining 10.8% of the variation in dose requirements. A regression model including *VKORC1* genotype and an indication of Warfarin therapy was a significant predictor of stable dose (p = 0.001). **Conclusion:** In Sudanese patients, Warfarin dose is influenced by *VKORC1-1639G>A* and *CYP2C9 430C>T* polymorphisms and therapeutic indication. Therefore, including pharmacogenetics data in the dosing algorithm may improve Warfarin therapy accuracy in Sudanese subjects.

Key words: Warfarin, pharmacogenetics, *CYP2C9*, *VKORC1*, polymorphisms, genotype, INR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Warfarin is the most commonly used oral anticoagulant worldwide[1]. It is used to prevent and treat thrombotic events in atrial fibrillation, cardiac valve replacement and Venous Thromboembolism (VTE)[1]. Warfarin exerts its anticoagulant effect by interfering with the bioavailability of vitamin K, which in turn obstruct the activation of vitamin K dependent clotting factors (II, VII, IX and X)[2].

Treatment with Warfarin is complicated by the wide inter and intra-individual variation of response to this drug. For this reason, Warfarin dose is adjusted by regular monitoring of anticoagulation status by laboratory determination of international normalized ratio (INR)[2]. Several factors were described to explain variation in the anticoagulation response to Warfarin therapy. Among these factors are age, gender, Body Mass Index (BMI), vitamin K intake, co-administered drug and disease status. Besides that, several genetic variations, mainly in *CYP2C9* and *VKORC1* genes, were investigated for their influence on Warfarin anticoagulation response[3,4].

Cytochrome P450 2C9 (*CYP2C9*) gene, which codes for the *CYP2C9* enzyme, was the first studied gene in this era. *CYP2C9* is a polymorphic enzyme that represents the main form of human liver *CYP450* that modulates the *in vivo* anticoagulant activity of Warfarin. Single Nucleotide Polymorphisms (SNPs) of the *CY2P2C9* gene have been reported to change the enzymatic activity of the hepatic enzyme, leading to an increased or decreased rate of Warfarin metabolism, which in turn raises or reduces the dose requirements, respectively[5]. Among 34 *CYP2C9* variant alleles, R144C (430C>T, rs 1799853), also called *CYP2C9*2* in exon 3 and I359L (1075A>C, rs1057910), also called *CYP2C9*3* in exon 7, have the most significant impact on Warfarin kinetics. They metabolize S-Warfarin with a capacity of approximately 12% and <5%, respectively, compared to the wild type (*CYP2C9*1*). Carriers of *2 and *3 alleles required lower doses of Warfarin and were at increased risk of major bleeding complications compared to wild type alleles[6]. In Sudan, carriers of *2, *5, *6 and *11 required a daily Warfarin dose which is 21% lower than the wild type allele[7].

The second gene candidate is the *VKORC1* gene, which codes for vitamin K epoxide reductase subunit one enzyme. This enzyme is responsible for vitamin K recycling and there represents the target of Warfarin therapy[2]. Among these gene variations,-1639 G>A (rs9923231) polymorphism located in 5' UTR of *VKORC1* gene promoter region was associated with significantly reducing Warfarin dose requirements for different ethnic groups[8]. In Sudan,-1639 G>A, 1173 C>T and additional three *VKORC1* SNPs were associated with lower Warfarin dose requirements[7].

Inappropriate Warfarin dosing may associate with a significant risk of bleeding due to over dosage or thrombosis due to insufficient dose[9]. Therefore, it is essential to identify factors that influence the dose requirement in each population, including the genetic ones. To the best of our knowledge, in Sudan, minimal data is available about the contribution of *CY2P2C9* and *VKORC1* genetic polymorphisms in the variation of Warfarin dose requirements. After that, the current study aimed to explore the influence of *CYP2C9* and *VKORC1* polymorphisms on Warfarin dose requirements among Sudanese subjects.

MATERIALS AND METHODS

Study area: This study was conducted in Sudan Heart Centre, Khartoum-Sudan in duration between 2019-2020. Patients receiving stable Warfarin therapy for different indications who attended the centre for follow-up during the study period were enrolled. Patients with medical conditions known to interact with Warfarin, like cancer and renal or hepatic insufficiency, were excluded. The purpose of the study was explained to all patients. an informed consent also was taken from each participant.

From 165 patients, a venous blood sample (1.8 mL) was collected in a Trisodium citrate tube and the Buffy coat was separated by centrifugation. DNA extraction was performed on Buffy coat samples using the Phenol chloroform method described by Sambrook and Russell[10] DNA samples were kept at-20°C until usage.

Genotyping: Genotyping of all samples for *CYP2C9 430C>T* and *VKORC1-1639G>A* polymorphism was performed by polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) as described by Natarajan *et al.*[11].

PCR reactions were performed in premix tubes (Intron Biotechnology, Korea), primers were from (Macrogen, Seoul, Korea), while thermocycling was performed in primus 96 (*peQlab*) PCR machine. Restriction enzymes from (New England BioLabs Inc, UK) were used to digest all PCR products. Sequences of primers, cycling conditions and restriction enzymes were shown in Table 1.

All PCR and digestion products were analyzed by electrophoresis on 2.5% agarose (iNtRON Biotechnology, Korea) gel stained with Ethidium bromide.

Warfarin dose calculation: The International Warfarin Pharmacogenetics Consortium (IWPC) algorithm was applied for Warfarin dose calculation. Variables for dose calculation included patients' demographics and genotypes of *VKORC1-1639G>A* and *CYP2C9 430C>T*.

Table 1: Primers, cycling conditions and restriction enzymes for all PCR-RFLP reactions

	CYP2C9 430C>T	*VKORC1-1639G>A*
Primers	F: 5'TCCTAGTTTCGTTTCTCTTCCTGT-3'	F: 5'GAGCCAGCAGGAGAGGGAAATAT-3'
	R: 5'ATAGTAGTCCAGTAAGGTCAGTGA-3'	R: 5'GTTTGGACTACAGGTGCCTGCC-3'
Cycling conditions		
Number of cycles	34	34
Denaturation	94°C for 1 min	94°C for 1 min
Annealing	60°C for 1 min	71°C for 30 sec
Extension	72°C for 1 min	72°C for 30 sec
Product size	221 bp	291 bp
Restriction enzyme	*Ava* II	*MSP* I

Statistical analysis: According to Hardy Weinberg equilibrium, the distribution of alleles was calculated with the Chi-square test using one degree of freedom (df), $p<0.05$ were considered deviated from the equation. Comparison of mean daily Warfarin dose between different genotype groups was calculated by Mann-Whitney U or Kruskal-Wallis tests.

The effect of patients' variables on Warfarin dose was measured. These variables include age, gender, comorbid disease, concurrent medications and an indication of Warfarin therapy. In addition, the effect of genetic factors on Warfarin dose was also measured. Univariate regression analysis was applied using each one of these factors as an independent variable to predict the mean daily Warfarin dose as a dependent variable. Then, a multiple regression analysis was performed to ascertain the combined influence of clinical and genetic factors as independent variables to predict the mean daily Warfarin dose. Finally, stepwise multiple regression analysis was performed to isolate independent variable(s) with the most significant influence on the Warfarin dose. A regression model for Warfarin dose prediction was developed. Pearson's correlation was used for comparison between pharmacogenetics' predicted dose and current dose.

For all tests, $p<0.05$ were considered statistically significant. All analysis was carried out using a statistical package for social sciences (SPSS) version 21 (IBM Japan Ltd., Tokyo, Japan).

RESULTS

A total of 165 patients (97 females and 68 males) who participated in this study received Warfarin therapy. The demographics and clinical characteristics of all patients were summarized in Table 2. Concerning the health status of participants, 11.5% of participants (19/165) had comorbid diseases, including hypertension (9 patients), Diabetes mellitus (5 patients), both of them (3 patients), Hyperthyroidism and hyperlipidemia (one patient each). About 20.6% of total patients receive medication alongside Warfarin, those drugs were stratified according to their effect on Warfarin to potentiate, inhibit, or have no effect on Warfarin action[12] Regarding Warfarin activators, five patients receive oral anti-diabetics, one patient receives Atorvastatin and another patient receives Aspirin. While regarding Warfarin inhibitors, five patients receive Furosemide and one patient receives an anticonvulsant agent.

Table 2: Descriptive statistics of studied population

Age: mean, SD, range (years)	42.2±14 (18-75)
Gender	
Male: N (%)	68 (41.2%)
Female: N(%)	97 (58.8%)
Indication for Warfarin therapy	
Mechanical valve replacement: N (%)	129 (78.1%)
Atrial fibrillation: N (%)	23 (13.9%)
Deep venous thrombosis: N (%)	13 (8%)
Mean daily Warfarin dose (mg/day+SD, range)	5.4±2.2 (1-13)
INR (Mean+SD, range)	3.4±1.9 (1-13.6)
*In therapeutic range: N (%)	105 (63.6%)
Out of therapeutic range: N (%)	60 (36.4%)
Comorbidity: N (%)	19 (11.5%)
On concurrent medication: N (%)	34 (20.6%)
Drug complication	
With bleeding: N (%)	5 (3.0%)
With thrombosis: N (%)	1 (0.6%)

*Patients who have INR values between (2.0-4.5) which represents the therapeutic range for different indications[13]

Genotyping results: Out of 165 patients' samples, DNA was extracted with good quality and sufficient quantity from 163 samples, while two samples gave poor DNA yield and they were excluded from further testing. For *CYP2C9* and out of 163 patients, 93.3% (152/163) were CC, while 6.7% (11/163) were CT. The frequency of the C allele is (0.966), while the T allele frequency is (0.033). Frequencies were following Hardy Weinberg equilibrium ($p = 0.655$).

Genotyping results for *VKORC1* were available for 143 samples, out of them, 50.3% (72/143) were GG, 36.4% (52/143) were GA and 13.3% (19/143) were AA. The frequency of the G allele is (0.685), while the A allele frequency is (0.314). Frequencies are following Hardy Weinberg equilibrium ($p = 0.06$).

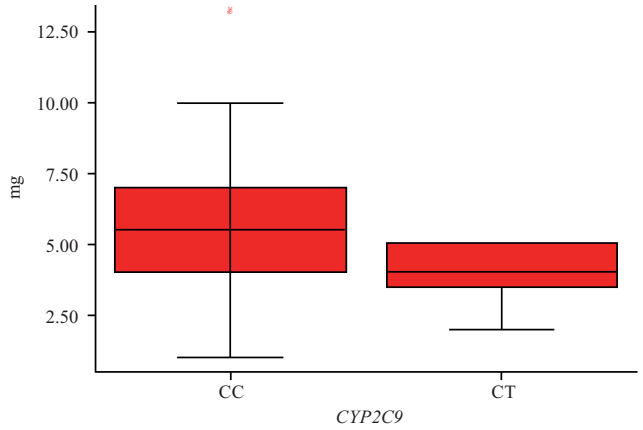

Fig. 1: Box and whisker plots showing mean daily Warfarin dose for *CYP2C9 430C>T* genotypes

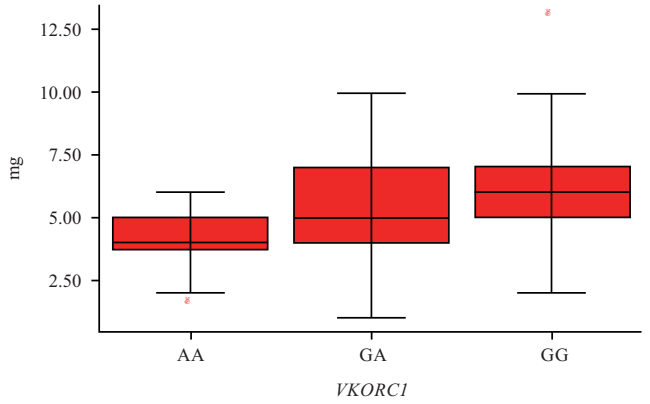

Fig. 2: Box and whisker plots showing mean daily Warfarin dose for *VKORC1-1639G>A* genotypes

Table 3: Results of univariate regression analysis

Predictors	R^2	Significance*
Age	0.026	0.101
Gender	0.000	0.926
Indication of Warfarin	0.046	0.028*
Comorbidity	0.021	0.144
Concurrent drug	0.003	0.575
CYP2C9	0.036	0.055
VKORC1	0.108	0.001**

*$p \leq 0.05$ is significant and **$p \leq 0.001$ is highly significant

Effect of *CYP2C9 430C>T* polymorphism on Warfarin dose: The frequencies of *CYP2C9* genotypes among 105 patients who had INR values within the therapeutic INR range were 93.3% (98/105) of the patients were homozygous for the wild type (CC) and 5.1% (5/105) were heterozygous (CT). In comparison, there were no genotype results obtained for the two patients. The mean daily Warfarin dose among the CC genotype group was (5.6±2.1 mg/day), while in the CT genotype group, it was (3.9±1.2 mg/day). Comparison between the two genotype groups using Mann Whitney U test revealed that *CYP2C9430C>T* polymorphism was associated with significantly lower Warfarin dose requirements ($p = 0.04$) compared with the wild type (Fig. 1). The *CYP2C9 430 T* variant allele was associated with a 30% reduction in Warfarin dose compared to the normal allele.

Effect of *VKORC1-1630 G>A* polymorphism on Warfarin dose: Regarding *VKORC1-1639* genotypes, out of 94 patients who had INR values within the therapeutic range, the frequencies of GG, GA and AA genotypes were 52.1% (49/94), 31.9% (30/94) and 15.9% (15/94), respectively. The mean daily Warfarin dose in the GG genotype group was (6.1±2.2 mg/day), while in GA/AA genotypes group, it was (4.8±1.9 mg/day). A comparison between these two genotypes groups using Mann Whitney U test revealed that *VKORC1-1639G>A* polymorphism was associated with significantly lower Warfarin dose requirements ($p = 0.004$) (Fig. 2). The *VKORC1-1639 A* variant allele was associated with a 21% reduction in the mean daily Warfarin dose compared to the wild type.

Factors influencing variation in Warfarin dose requirements: The univariate regression analysis for the effect of clinical and genetic factors on the mean of the daily Warfarin dose revealed that among tested variables, the highest contribution to the variation of Warfarin dose was attributed to *VKORC1* genotype (10.8%, $p = 0.001$) (Table 3).

Multiple regression analysis revealed that 30.1% of the variation in Warfarin dose requirements was explained by clinical factors (age, gender, indication for Warfarin therapy, comorbid disease and concurrent medication) and genetic polymorphisms, including genetic polymorphisms *CYP2C9 430C>T* and *VKORC1-1639G>A*. The regression model including all the above factors significantly predicted Warfarin dose in this population (F score = 3.159, $p = 0.003$). Among these factors, *VKORC1* genotype and indication for Warfarin therapy were the most significant predictors of Warfarin dose ($p = 0.006$ and 0.022, respectively) (Table 4). In stepwise regression analysis, only the *VKORC1* genotype and indication for Warfarin therapy were retained in the final model. Those two factors explained 17.7% ($R^2 = 0.177$) of the variation in daily Warfarin dose, with the *VKORC1* genotype being the major predictor in this study population ($R^2 = 0.104$). The model, including the *VKORC1* genotype and an indication of Warfarin therapy, significantly predicted Warfarin dose (F score = 7.856, $p = 0.001$). Results of stepwise regression analysis were shown in Table 5.

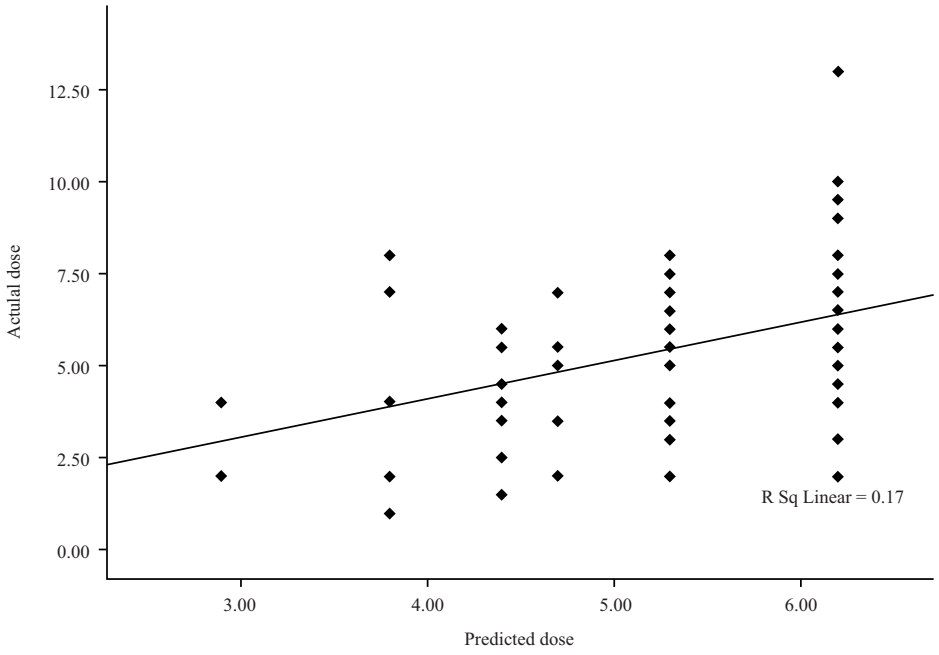

Fig. 3: Relationship between Warfarin doses and the actual daily Warfarin doses among patients

Table 4: Results of multiple regression analysis

	Un-standardized coefficients		Standardized coefficients		
Independent variables	B	Std. Error	Beta	T	Sign
Constant	3.431	1.770		1.938	0.057
Years	-0.018	0.017	-0.127	-1.082	0.283
Gender	-0.635	0.420	-0.157	-1.513	0.135
Indication	1.417	0.606	0.217	2.337	0.022*
Comorbidity	-1.551	0.836	-0.287	-1.856	0.068
Concurrent	0.622	0.697	0.139	0.891	0.376
CYP2C9	1.568	0.792	0.214	1.979	0.052
VKORC1	0.853	0.298	0.311	2.866	0.006*

*$p \leq 0.05$ is significant

Table 5: Coefficients of stepwise regression models

	Un-standardized coefficients		Standardized coefficients		
Models	B	Std. Error	Beta	T	Sign
1 (constant)	4.176	0.462		9.031	0.000**
VKORC1	0.885	0.301	0.323	2.937	0.004*
2 (constant)	2.986	0.647		4.617	0.000**
VKORC1	0.899	0.291	0.328	3.090	0.003*
Indication	1.412	0.556	0.270	2.539	0.013*

*$p \leq 0.05$ is significant and **$p \leq 0.001$ is highly significant

Comparisons between regression predicted Warfarin dose with the actual and IWPC predicted doses: The equation obtained from the stepwise regression model was used to calculate the Warfarin dose for 94 patients who had INR values within the therapeutic range and available *VKORC1* genotype. The doses calculated using the regression equation ranged between 2.9 and 6.2 mg (Mean 5.3±.85 mg), while predicted Warfarin doses calculated by (IWPC) dosing algorithm ranged between 2.5 and 7.0 mg (Mean 5.0±1.2 mg). The regression predicted doses obtained in this study, showed significant positive correlations with the actual doses on one hand and with (IWPC) predicted doses on the other hand (both $p = 0.000$) (Fig. 3).

DISCUSSION

This study aimed to investigate the contribution of genetic polymorphisms to the variability of Warfarin dose requirements in Sudanese patients. Firstly, regarding Warfarin doses and INR values, participants in this study showed a wide range of Warfarin dose requirements (1.0-13.0 mg) and INR values (1.0-13.6). These values give further evidence that Warfarin is a drug with wide inter-subject variability in dose requirements and anticoagulation effect respectively, which was following previous reports on different ethnic groups.

The impact of clinical factors on Warfarin dose requirements was evaluated in this study. Among the tested variables, the only indication of Warfarin therapy significantly impacted Warfarin dose ($p = 0.028$). The other factors, including patient's age, gender, comorbid diseases and concurrent medications, had a minor effect on Warfarin dose requirements. This finding was following the previous findings in Sudanese patients[7]. This finding suggested that further clinical factors may influence Warfarin dose.

On the other hand, this finding disagreed with previous observation of associations between old age and the female gender with lower Warfarin dose requirements[14]. In this study, the age factor explained only 2.6% of dose variation while the gender factor had no impact on Warfarin dose. This discrepancy could be explained by the relatively younger ages of participants in this study (Mean 42.2 years), while the effect of gender might be diluted by the effects of other clinical and genetic factors.

Regarding minor allele frequencies (MAFs) of studied SNPs observed in this study about other populations, *CYP2C9*2* MAF is more significant than what had been observed in African and Asian but is lower than in European and Latino populations. Regarding *VKORC1-1639G/A*, the MAF is greater than what had been observed in Africans but it's lower than in Asian, European and Latino populations[15,16].

In the present study, 3.6% of the variation in Warfarin dose requirements was attributed to *CYP2C9 430C>T* (*CYP2C9*2*) polymorphism, which is associated with a 30% reduction in Warfarin dose compared with the wild type. This finding was comparable to the previous finding in Sudanese, which showed that *CYP2C9*2*, *5 and *6 variant alleles are associated with a 16-33% reduction in Warfarin dose compared with the wild type[7]. On the other hand, the percentage value of *CYP2C9*2* polymorphism effect on Warfarin dose observed in this study is lower than what had been observed in Europeans as *CYP2C9*2* and *3 polymorphisms explained 15% of Warfarin dose variation in these populations[17]. This discrepancy could be attributed to the lower frequency of this SNP in Sudanese compared with Europeans[15].

The percentage of variability in Warfarin maintenance dose accounted for by the *VKORC1-1639* genotype was 10.8%. This value was following the previous finding in Sudanese subjects, which showed that three *VKORC1* SNPs (including-*1639G/A*) explained 14-16% of dose variation[6]. On the other hand, this value is lower than what had been observed in European as 15-30% of Warfarin dose variation was explained by *VKORC1* haplotypes (including-1639G/A)[17]. In the Asian population, this polymorphism explained 29% of dose variation and this is related to the frequency of the-1639 A allele in Asians, which is substantial (90%) compared to Sudanese[8].

The main finding of this study was that 30.1% of the variation in Warfarin dose requirements in Sudanese patients was explained by combined effects of genetic factors including *CYP2C9* and *VKORC1* polymorphisms and clinical factors an indication of Warfarin therapy, age, gender, comorbid diseases and concurrent medications. This percentage value was slightly lower than the reported value in Sudanese, which showed that genetic and non-genetic factors explained 36% of the variation in Warfarin dose requirements. This difference could be attributed to the fewer independent variables included in this study compared with that study[7]. All values mentioned above were much lower than what had been observed in Indians (67%)[18] and Egyptians (30%)[19]. However, they are close to the variability explained by such factors in Chinese (37%)[20] African-Americans (29%)[21].

Finally, the Warfarin pharmacogenetic dosing algorithm developed in this study showed a positive correlation between the actual stable Warfarin dose and the IWPC pharmacogenetics predicted dose. These correlations indicated that the inclusion of pharmacogenetics data in Warfarin dosing algorithms might increase the predictive power of such algorithms. This observation was by the findings of prospective trials like the genetics informatics trial (GIFT) and European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial[22,23]. These studies reported that pharmacogenetics algorithms were associated with fewer out of range INRs and more extended periods in the therapeutic range compared to standard dosing algorithms. Although another major trial called clarification of oral anticoagulation through genetics (COAG) showed no significant difference in the performance of the pharmacogenetics algorithm and the clinically guided dosing algorithm ($p = 0.91$)[24]. However, the value of pharmacogenetics data in Warfarin dosing algorithms is well recognized.

As a result of the patients' lack of understanding of the importance of the study despite attempts to provide a full and brief explanation, most patients have chronic diseases and feel annoyed by the repeated sampling of them. A prospective study on Sudanese patients could evaluate the pharmacogenetic dosing algorithm developed in this study. Moreover, additional polymorphisms that might affect Warfarin dose requirements in Sudanese patients need to be investigated.

CONCLUSION

In Sudanese patients, Warfarin dose is significantly influenced by *VKORC11639G>A* and *CYP2C9 430C>T* polymorphisms and the indication of Warfarin treatment. 3.6% of the variation in Warfarin dose requirements was attributed to *CYP2C9 430C>T* (*CYP2C9*2*) polymorphism, which is associated with a 30% reduction in Warfarin dose compared with the wild type. The other factors, including patient's age, gender, comorbid diseases and concurrent medications, had a minor effect on Warfarin dose requirements.

SIGNIFICANCE STATEMENT

This study discovered that *VKORC1 1639G>A* and *CYP2C9 430C>T* polymorphisms have a significant effect on Warfarin dose. These findings imply that incorporating pharmacogenetics data into the dosing algorithm may improve Warfarin medication accuracy in Sudanese patients. This research will help researchers in identifying crucial locations of additional polymorphisms that may impact Warfarin dose requirements.

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