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ORIGINAL RESEARCH ARTICLE

Validation of a Proposed Warfarin Dosing Algorithm Based on the Genetic Make-Up of Egyptian Patients

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Abstract

Background Warfarin is the most frequently prescribed oral anticoagulant worldwide. Due to its narrow therapeutic index and inter-patient variability in dose requirement, this drug has been considered an ideal target for personalised medicine. Several warfarin dosing algorithms have been proposed to tailor the warfarin dosage in the European, Asian and African-American populations. However, minimal interest was directed towards Middle East countries. The factors affecting warfarin dose requirement could be different in patients from different geographical and ethnic groups, limiting the value of published dosing algorithms.

Objective The first objective of this study was to examine the contribution of genetic and nongenetic factors on the variability of warfarin dose requirements in the Egyptian population using an easy, cost-effective and rapid analysis of vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 (*CYP*) *2C9* single nucleotide polymorphism (SNP) genotyping of patients. A second objective was to develop and validate an algorithm for warfarin dose prediction that is tailored to Egyptian patients.

Methods Eighty-four patients, 41 males and 43 females, with a median (25th–75th percentiles) age of 39 (31–48) years were recruited in this study. Fifty patients whose international normalised ratio (INR) was in the range of 2–3 were allocated to a study cohort. SYBR Green-based multiplex allele-specific real-time PCR was used for genotyping of *CYP2C9* (1075A>C) and *VKORC1* (1173C>T) polymorphisms. Linear regression analysis, including the variables age, gender, *CYP2C9* and *VKORC1* SNP genotypes, was run to derive the best model for estimating the warfarin dose that achieves an INR of 2–3. The new warfarin dosing algorithm was examined in a second cohort of patients ($n = 34$) to check its validity. The predicted dose requirements for a subgroup of our patients were calculated according to Gage and International Warfarin Pharmacogenetics Consortium (IWPC) algorithms available at http://www.warfarindosing.org.

Results In the study cohort, warfarin dose/week in *VKORC1* TT subjects was statistically significantly lower than in *VKORC1* CC/CT subjects ($p = 0.032$), while there was no statistically significant difference in warfarin dose/week between *CYP2C9**1*1 and *1*3 ($p = 0.925$). A multivariate stepwise linear regression analysis revealed that age and *VKORC1* had independent and significant contributions to the overall variability in warfarin dose with a p-value = 0.013 and 0.042, respectively. Maintenance dose (mg/week) = 65.226 − 0.422 × (age) − 9.474 × (*VKORC1*). The estimated regression equation was able to account for 20.5 % of the overall variability in warfarin maintenance dose. A significant positive correlation, with sufficient strength, was observed between the predicted warfarin dose and the actual prescribed dose ($r = 0.453$, $p = 0.001$). In the validation cohort, after application of the dosing algorithm, correlation between predicted and actual dose was statistically significant ($p = 0.023$). The equation was particularly successful among patients with a dose ≥35 mg/week. The correlation

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coefficient between the actual and predicted doses for IWPC and Gage were 0.304 and 0.276, respectively. When compared with our algorithm ($r = 0.279$), the difference was non-significant: $p = 0.903$ and 0.990, respectively.
Conclusion *VKORC1* (1173C>T) contributes to the warfarin dose variability. Patients' age and genetic variants of *VKORC1* account for nearly 20.5 % of the variability in warfarin dose required to achieve an INR of 2–3. The success of a prediction equation based on these variables was proved in a different cohort: the predicted dose correlated significantly with the maintenance dose and the equation was more successful among patients with a dose ≥35 mg/week. The results of the warfarin algorithm we developed were comparable with those of the IWPC and Gage algorithms with the advantage of using one SNP (*VKORC1* 1173C>T) only. This represents an economic advantage in our community. Replication of this study in a larger cohort of patients is necessary before translation of this knowledge into clinical guidelines for warfarin prescription.

1 Introduction

Warfarin is the most frequently prescribed oral anticoagulant worldwide [1]. Nevertheless, the US FDA rates warfarin among the top ten drugs with the greatest number of serious adverse reactions [2], mostly due to its narrow therapeutic window and high inter- and intra-individual variability in dose requirements. For these reasons, careful laboratory monitoring is necessary to maintain the international normalised ratio (INR) within the therapeutic range [3].

A number of factors have been identified to influence the anticoagulation effect of warfarin therapy, including age, sex, body weight, co-morbidity, concurrent medications, dietary intake, patient compliance level and genetic factors [4]. Warfarin is a racemic mixture of *S*- and *R*-enantiomers. The *S*-enantiomer is associated with 60–70 % of warfarin's anticoagulant response, while the *R*-enantiomer accounts for 30–40 % of warfarin's anticoagulant effect [5]. The *S*-enantiomer is metabolised at therapeutic concentration predominantly by cytochrome P450 (CYP) 2C9 [5]. The wild-type allele is *CYP2C9**1 [5]. At least 33 clinically relevant variants of *CYP2C9* (*2 through to *34) have been documented [6]. The most common variants *CYP2C9**2 (430C>T, R144C, rs1799852) and *CYP2C9*3* (1075A>C, I359L, rs1057910) generate enzymes with impaired hydroxylation of *S*-warfarin due to amino-acid changes, and several studies have shown that these variants have an effect on warfarin dose requirement [7–9].

Warfarin is a specific inhibitor of the vitamin K epoxide reductase (VKOR) encoded by the VKOR complex subunit 1 (*VKORC1*) gene [10]. Warfarin exerts its anticoagulant effects by preventing the ability of *VKORC1* to regenerate reduced vitamin K from its epoxide form [11]. Reduced vitamin K is an essential cofactor for γ-glutamylcarboxylase (GGCX), the enzyme catalysing the post-translational γ-glutamyl carboxylation of the vitamin K-dependent clotting factors, II (prothrombin), VII, IX and X. Thus, warfarin prevents the functional maturation of vitamin K-dependent clotting factors, leading to reduced coagulation [12]. Genetic variations in *VKORC1* are associated with altered sensitivity to warfarin [13]. Two single nucleotide polymorphisms (SNPs) of *VKORC1* were found to estimate warfarin dosing phenotypes: −1639G>A (rs9923231) promoter polymorphism and 1173C>T (rs9934438) intronic polymorphism [9]. Studies of white and Asian patients have reported that *CYP2C9* and *VKORC1* polymorphisms influence the maintenance dose of warfarin [14, 15], but they appear to contribute to less than half of the inter-individual variability in the dose-response relationship to oral anticoagulants [14]. Several warfarin dosing algorithms have been proposed to tailor the warfarin dosing in the European, Asian and African-American populations. However, minimal interest was directed towards Middle East countries. The factors affecting warfarin dose requirement could be different in patients from different geographical and ethnic groups, limiting the value of published dosing algorithms.

The first aim of our study was to investigate the contribution of genetic and nongenetic factors on the variability of warfarin dose requirements in the Egyptian population using an easy, cost-effective and rapid analysis of *VKORC1* and *CYP2C9* genotyping of patients. The second objective was to develop and validate an algorithm for warfarin dose prediction that is tailored to Egyptian patients.

2 Subjects and Methods

2.1 Subjects

To create the study group, from October 2010 to November 2011, we investigated patients referred to Kasr Al-Ainy University Hospital (Cairo, Egypt) outpatient clinic for warfarin (Marevan™; GlaxoSmith Kline, Cairo, Egypt) therapy monitoring. The average number of patients attending the clinic is 60 patients/week. Dosing of warfarin is not computer assisted. The physicians prescribe warfarin following the empiric dosing method. Time in therapeutic range is 75 % on average. We selected 50 patients [23 males and 27 females, mean (standard deviation) age: 39.16 (12.00) years] who satisfied the following inclusion criteria: age >18 years, oral

anticoagulation therapy (OAT) lasting for at least 3 months, and stable warfarin dose. A patient was considered to be on a stable warfarin dose if he or she had at least three consecutive INRs in the therapeutic (2–3) range for the same daily maintenance dose after at least 3 months of therapy. Exclusion criteria included cigarette smokers, subjects with abnormal liver function tests (alanine aminotransferase and aspartate aminotransferase $\geq$3 times the upper limit of normal) or thyroid function tests (definite hypothyroidism or hyperthyroidism), malnutrition, decompensated heart failure, bleeding diathesis, those receiving any drug known to have a major interaction with warfarin [e.g. amiodarone, statins (HMG-CoA reductase inhibitors), omeprazole, NSAIDs, anticonvulsants, sulfonamides, rifampin, azole antifungals and vitamin preparations containing vitamin K] and known noncompliance with OAT. Clinical data were obtained from the medical records compiled at the time of warfarin initiation after an interview by a physician. In the medical record, the presence of co-morbidities and the use of drugs apart from warfarin were also noted. Patients were excluded if clinical data were incomplete. Patients in the validation group ($n = 34$) were selected with the same inclusion/exclusion criteria used for the study group. The study was approved by the local Research Ethics Committee, and all patients provided informed consent.

2.2 Methods

For each selected patient, prothrombin time (PT) was measured by standard methods using the STA Compact® Hemostasis System (Diagnostica Stago, France). Genomic DNA was extracted from EDTA anticoagulated blood by means of QIAamp DNA blood Mini kit (Qiagen, Germany).

2.2.1 *Genotyping Assay for Detection of Cytochrome P450 2C9 (1075A>C) (rs1057910) and Vitamin K Epoxide Reductase Complex Subunit 1 (1173C>T) (rs9934438) Polymorphisms by Using SYBR Green-Based Multiplex Allele-Specific PCR*

CYP2C9 and *VKORC1* genotyping were determined using SYBR Green-based multiplex allele-specific PCR and consequent melting curve analysis as previously described [16] by using StepOne™ Real-Time PCR Systems (Applied Biosystems, USA). Primers' sequences are shown in Table 1 and they were designed by The Midland Certified Reagent Company (Texas, USA). For genotyping *VKORC1* 1173C>T locus, a 27-nt GC-tail was added to the 5′ end of the forward specific-primer that could only amplify the allele where the polymorphic site has a *T* residue. For the *CYP2C9**3 polymorphism, a 28-nt GC-tail was added to the 5′ end of the forward specific-primer that could only amplify the *1 allele which has an *A* residue at position 1,075.

The 25 μL reaction mixture for both *CYP2C9* and *VKORC1* included 12.5 μL SYBR Green Mastermix (QuantiTect SYBR Green PCR Kits, Qiagen, USA) and 40 ng DNA. For *CYP2C9*, 0.6 μM C-primer forward, 0.15 μM A-primer forward (GC-tail) and 0.15 μM primer reverse were used. For *VKORC1*, 0.2 μM C-primer forward, 0.15 μM T-primer forward (GC-tail) and 0.15 μM primer reverse were used.

PCR amplification of *CYP2C9* and *VKORC1* was performed under the same conditions as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s and extension at 72 °C for 15 s, and a final extension at 72 °C for 2 min. After amplification, melting curve analysis was performed by heating the reaction mixture from 60 to 95 °C at a rate of 0.5 °C/min. The StepOne™ Real-Time PCR Systems automatically calculated the negative derivative of the change in fluorescence and generated a melting curve for each sample, as shown in Fig. 1. We attempted to develop a pharmacogenetic algorithm, choosing one *CYP2C9* variant and one *VKORC1* SNP, to reduce the costs to a minimum, and examine their impact on warfarin dose variation. It was decided to choose *CYP2C9**3 and not *CYP2C9**2 based on the fact that *CYP2C9**3 has more effect (−21 to −49 %) compared with *CYP2C9**2 (−14 to −20 %), as previously reported [17].

2.2.2 *Application of International Warfarin Pharmacogenetics Consortium and Gage Algorithms to Compare Results of these Algorithms to the One Created in the Present Work*

The predicted dose requirements for a subgroup of our patients for whom bodyweight and height were available ($n = 35$) were calculated according to Gage [18] and International Warfarin Pharmacogenetics Consortium (IWPC) algorithms [19] available at http://www.warfarindosing.org [20]. Parameters required for warfarin dose prediction by the two dosing algorithms were *CYP2C9* and *VKORC1* genotypes, age, height, bodyweight, gender, race, ethnicity, concomitant medications, target INR, smoking status and warfarin indication. Results of *VKORC1* 1173 C>T (rs9934438) were imputed to *VKORC1* −1639G>A (rs9923231) as previously described [19].

2.2.3 *Statistical Analysis*

Numerical data were summarised as mean ± standard deviation when parametric and as median (25th–75th

Table 1 Primers' sequences for *CYP2C9* 1075A>C and *VKORC1* 1173C>T polymorphisms

SNP position	Primers' sequences (5′→3′)
VKORC1	FT: GCCGAGGAGGAGCCGAGGGAGCGAGCCGCCAGGAGATCATCGAg**T**
1173C>T	FC: GCCAGGAGATCATCGAa**C**
	R: CACCTGGGCTATCCTCTG
CYP2C9	FA: GGGAGCCCGACCACGGACGGAGGCACCGCACGAGGTCCAGAGATACA**A**
1075A>C	FC: CACGAGGTCCAGAGATAC**C**
	R: GGAATGAGATAGTTTCTGAATTTAAT

The polymorphic base is in bold font, the 3′ mismatch is in lowercase, and the GC-tail is underlined

CYP cytochrome P450, *F* forward primer, *R* reverse primer, *VKORC1* vitamin K epoxide reductase complex subunit 1

percentiles) when non-parametric. Differences between groups were detected using Student's *t*-test for the former and Mann–Whitney test for the latter. Nominal data were summarised as number (percentage). Multivariate stepwise linear regression analysis was conducted using age, gender, *VKORC1, CYP2C9* and combined *VKORC1* and *CYP2C9* as covariates in order to reach the minimum combination that can be used to predict the warfarin dose suitable to achieve an INR of 2–3. Correlation analysis was used to examine the strength of association between the predicted warfarin dose and the actual prescribed dose. The performance of the algorithm was assessed in two dose groups: participants requiring a dose <35 mg/week and those requiring a dose ≥35 mg/week for stable therapeutic anticoagulation. The threshold of 35 mg/week was chosen as the usual starting dose is 5 mg/day. Statistical analysis was run on SPSS for Windows, release 17.0 (SPSS Inc., Chicago, IL, USA). *p*-Values <0.05 were considered statistically significant.

3 Results

3.1 Demographic Data of the 84 Studied Subjects

Demographic data of the 84 studied subjects were subdivided into the study and validation cohorts. The median (25th–75th percentiles) age of the 84 patients was 39 (31–48) years. Forty-one were men and 43 were women. Most of the patients (75 %) received warfarin for valvular diseases. There was no significant difference in the demographic data of the two cohorts of patients as shown in Table 2.

3.2 Associations with Warfarin Dose

Our results show that the maintenance dose decreases, on average, by 0.4 mg/week for every 1 year increase in age. The warfarin weekly doses prescribed to patients were statistically significantly lower in *VKORC1* TT when compared to *VKORC1* CC/CT subjects in the study cohort ($p = 0.032$). There was no statistically significant difference in warfarin dose/week between *CYP2C9**1*1 and *1*3 ($p = 0.925$) as shown in Table 3, and the Online Resource (Supplementary Figure IA and B).

3.3 Warfarin Dosing Algorithm

To assess the effective factors for warfarin maintenance dose, a multivariate stepwise linear regression analysis was conducted with respect to age, gender, *VKORC1, CYP2C9* and combined *VKORC1* and *CYP2C9* in the study group ($n = 50$). Analysis revealed that age and *VKORC1* had an independent and significant contribution to the overall variability in warfarin dose ($p = 0.013$ and 0.042, respectively); other factors didn't contribute to the prediction of warfarin dose ($p > 0.05$). The regression equation for warfarin is as follows:

$$\text{Maintenance dose}\,(mg/\text{week}) = 65.226 - 0.422 \times (\text{age}) - 9.474 \times (VKORC1),$$

where age is in years and *VKORC1* is coded as 1 for TT and 0 for CT or CC. The estimated regression equation was able to account for 20.5 % ($R^2 = 0.205$) of the overall variability in warfarin maintenance dose. Age accounted for 13.2 % while *VKORC1* polymorphism accounted for the extra 7.3 % of variation. A significant positive correlation, with sufficient strength, was observed between the predicted warfarin dose and the actual prescribed dose ($r = 0.453$, $p = 0.001$), as shown in Fig. 2.

This dosing algorithm was assessed in a second unrelated group of 34 patients on warfarin therapy and with stable control of anticoagulation (validation cohort); patient characteristics and demographics are shown in Table 2. Correlation between predicted and actual dose in the validation cohort was statistically significant (Spearman's rho = 0.388, $p = 0.023$). The paired difference between the actual and predicted doses was statistically non-significant (Wilcoxon Signed Ranks Test, $p = 0.317$). The equation was more successful among patients with a dose ≥35 mg/week, where the predicted dose was less than 25 % different from the actual dose in 11 of 16 (68.8 %).

Table 2 Demographic data of the 84 studied subjects

	Total group ($n = 84$)	Study cohort ($n = 50$)	Validation cohort ($n = 34$)	p-Value[a]
Gender (male)	41 (48.8 %)	23 (46.0 %)	18 (52.9 %)	0.532
Age (years)	39.0 (31.0–48.0)	38.0 (30.8–44.2)	43.5 (33.8–52.8)	0.167
Dose/week (mg)	38.5 (30.4–49.0)	42.0 (28.0–49.0)	35.0 (31.1–49.0)	0.832
INR	2.31 (2.09–2.69)	2.3 (2.08–2.49)	2.42 (2.08–2.81)	0.170
VKORC1 1173C>T				
TT	57 (67.9 %)	38 (76 %)	19 (55.9 %)	0.053[b]
CT	7 (8.3 %)	1 (2 %)	6 (17.6 %)	
CC	20 (23.8 %)	11 (22 %)	9 (26.5 %)	
CYP2C9 1075A>C				
*1*1	70 (83.3 %)	43 (86 %)	27 (79.4 %)	0.426[c]
*1*3	10 (11.9 %)	7 (14 %)	3 (8.8 %)	
*3*3	4 (4.8 %)	0 (0 %)	4 (11.8 %)	
Indication of warfarin				
Valvular heart diseases	63 (75 %)	42 (84 %)	21 (61.8 %)	
Coronary heart diseases	2 (2.4 %)	1 (2 %)	1 (2.9 %)	
Deep venous thrombosis	12 (14.2 %)	6 (12 %)	6 (17.6 %)	
Atrial fibrillation	5 (6 %)	1 (2 %)	4 (11.8 %)	
Others[d]	2 (2.4 %)	0 (0 %)	2 (5.9 %)	
Co-morbidities				
No co-morbidity	47 (56 %)	34 (68 %)	13 (38.2 %)	
Hypertension	31 (36.8 %)	12 (24 %)	19 (55.9 %)	
Diabetes mellitus	3 (3.6 %)	3 (6 %)	0 (0 %)	
Congestive heart failure	1 (1.2 %)	1 (2 %)	0 (0 %)	
Arrhythmia	2 (2.4 %)	0 (0 %)	2 (5.9 %)	

Qualitative data are represented as frequency (percentage), while quantitative data are represented as median (25th–75th percentiles)

CYP cytochrome P450, *INR* international normalised ratio, *VKORC1* vitamin K epoxide reductase complex subunit 1

[a] p-Value is for comparison between study and validation cohorts

[b] TT vs. CT/CC

[c] *1*1 vs. *1*3/*3*3

[d] Others include aneurysm and Pace-maker

When the predicted dose for a subgroup of our patients for whom bodyweight and height were available ($n = 35$) was calculated using IWPC and Gage algorithms, the correlation coefficient between the actual and predicted doses was 0.304 and 0.276, respectively. When compared with our algorithm ($r = 0.279$), the difference was non-significant: $p = 0.903$ and 0.990, respectively.

4 Discussion

Performing genotyping of *CYP2C9* and *VKORC1* is recommended among patients before initiation of warfarin [21]. In the present study, an algorithm for warfarin dosing was suggested in a cohort of patients who had achieved a therapeutic INR of 2–3. The algorithm was subsequently validated in a second cohort of patients.

The results of the present study show that the patients' age and genetic variants of *VKORC1* demonstrate significant correlations with warfarin dose and account for nearly 20.5 % of the variability in warfarin dose required to achieve an INR of 2–3. Age contributed 13.2 %, while *VKORC1* genotypes contributed 7.3 % of the overall variability in warfarin maintenance dose. The results of our developed warfarin algorithm were comparable with those of the IWPC and Gage algorithms with the advantage of using one SNP (*VKORC1* 1173C>T) only. This represents an economic advantage in our community.

Recently, predictions of warfarin dose by multivariate analyses have been reported in several populations. In other

Table 3 Comparison of warfarin dose/week within the *VKORC1* and *CYP2C9* genotypes in the study cohort

	VKORC1 1173C>T			*CYP2C9* 1075A>C		
	TT ($n = 38$)	CT/CC ($n = 12$)	p-Value	*1*1 ($n = 43$)	*1*3 ($n = 7$)	p-Value
Dose/week (mg)	38.90 ± 13.00	49.50 ± 18.00	0.032	41.58 ± 14.58	41.0 ± 18.7	0.925

All data are presented as mean ± standard deviation

CYP cytochrome P450, *VKORC1* vitamin K epoxide reductase complex subunit 1

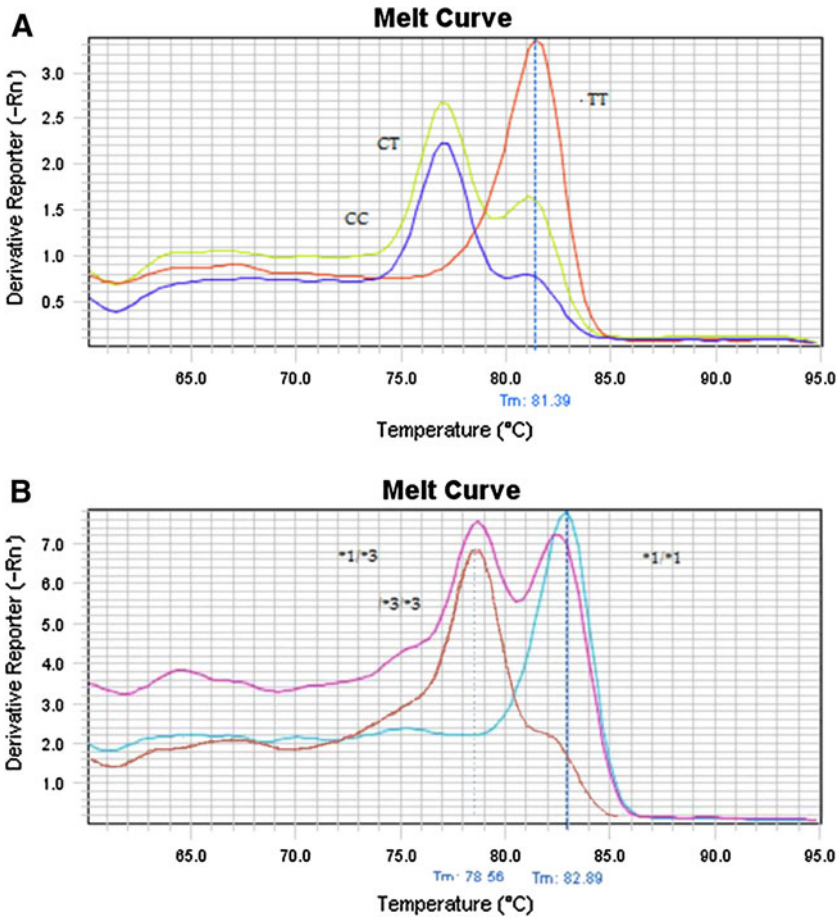

Fig. 1 **a** Melting curves for three different genotypes of vitamin K epoxide reductase complex subunit 1 (*VKORC1*) 1173C>T; profile with a single peak at 77.07 °C representing homozygote of CC, profile with a single peak at 81.39 °C representing homozygote of TT, and profile with a double peak at 77.07 and 81.39 °C representing heterozygote of CT. **b** Melting curves for three different genotypes of cytochrome P450 (*CYP*) *2C9* (1075A>C); profile with a single peak at 82.89 °C representing homozygote of AA (*1*1), profile with a single peak at 78.56 °C representing homozygote of CC (*3*3) and profile with a double peak at 82.89 and 78.56 °C representing heterozygote of AC (*1*3)

reports, R^2 ranged from 54 to 63 % [15, 22]. Comparison of R^2 in the above-mentioned studies may not be possible because the criteria-considered covariates are different in each study. The IWPC [19] ($n = 4{,}043$ participants) reported that the contribution of each tested variable (age, *VKORC1* and *CYP2C9*) in their proposed pharmacogenetic algorithm were 6.75, 13.3 and 7.2 %, respectively.

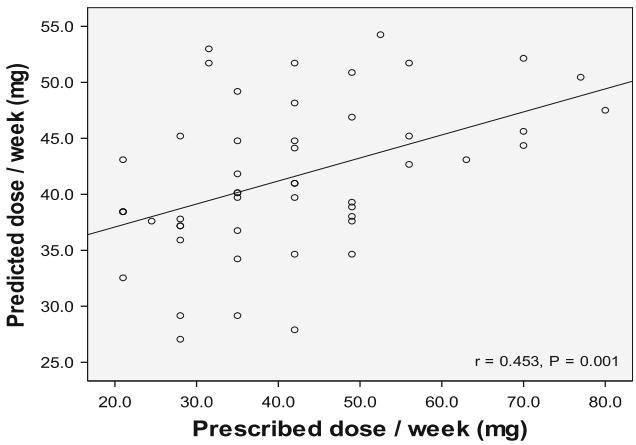

Fig. 2 Correlation between the prescribed and the predicted dose/week in the study cohort ($n = 50$)

Suriapranata et al. [23] concluded that non-genetic factors (age, bodyweight and height) contributed by 5.9 %, while *VKORC1* −1639G/A and *CYP2C9* rs17847036 accounted for 8.4 %, with a total contribution of 15.4 % to warfarin reactivity. Cho et al. [15] reported that covariates consisting of *VKORC1* 1173C>T SNP, age, BSA and *CYP2C9* genetic variants accounted for 58.2 % of the overall variability. Herman et al. [24] reported that covariates consisting of bodyweight, age, concomitant drug influencing warfarin metabolism and *CYP2C9* polymorphisms accounted for 37 % of the overall variability. Rusdiana et al. [25], found that the age and genetic variants of *CYP2C9* and *VKORC1* affected the variability of the warfarin response and was able to explain approximately 19.6 % of the PT-INR as a pharmacodynamic index and 16.3 % of the concentration of *S*-warfarin concentration as a pharmacokinetic index, in the setting of fixed low-dose warfarin therapy in the Indonesian population. As regards previous Egyptian studies, Shahin et al. [26] concluded that 31 % of variability in warfarin dose was explained by a combination of genetic and nongenetic factors; age contributed by 8.11 %, while *CYP2C9* genetic variants accounted for 5.17 % of the overall variability. El Din et al. [27] reported that all variables together (age, sex,

bodyweight, *VKORC1* and *CYP2C9**1/*2, *1/*3 and *2/*2 haplotypes) accounted for 61.3 % of the overall variability in warfarin dosages, while *VKORC1* (1173C>T) accounted for 31.7 % and *CYP2C9* accounted for 15.6 %. The variable contribution of the same SNP in the warfarin dosing algorithm in different ethnic populations may be attributed to different studied cohort sizes and ethnic differences, e.g. the *VKORC1* 1173TT genotype frequency (67.9 %) among our studied Egyptians was significantly more prevalent than that among Caucasians (12.5 %) [28].

In our study, the predicted dose achieved ideal estimation (less than 25 % difference from actual dose) in 47.1 % of patients in the validation cohort. Our findings were in agreement with the results reported by the IWPC [19] study, who reported that the predicted dose, calculated by a pharmacogenetic algorithm, showed an ideal estimation (20 % difference) in approximately 46 % of the validation cohort ($n = 1{,}009$). An interesting finding was the great increase in predictive accuracy among patients requiring a warfarin dose ≥35 mg/week (68.8 %). The good performance of the new algorithm in the upper dose range may not be useful to reduce the bleeding risk associated with warfarin therapy; however, it facilitates the detection of patients who need higher doses than the usual empirical starting dose. For these patients, identifying the dosage necessary to reach the therapeutic range may be cumbersome in current clinical practice, with the risk of inadequate anticoagulation and possible adverse thromboembolic events in the initial phase of the oral anticoagulant therapy, and with longer hospital stay.

The contribution of age and *VKORC1* 1173 SNP accounted for only 20.5 % of warfarin dose variability in our study cohort, while 79.5 % of the variability in dose requirement is left unaccounted for. The percentage of variation is relatively low compared with other models because besides *VKORC1* 1173C>T and *CYP2C9**3 variants, there exist other SNPs, in both the *VKORC1* and *CYP2C9* genes as well as other genes, that control pharmacokinetics and pharmacodynamics of warfarin which may have an impact on warfarin dose variability and need to be examined in the Egyptian population. By increasing the number of SNPs studied, the contribution of each SNP, even if it were of small value, would have a cumulative effect that would increase the percentage of variation. Height, bodyweight or body surface area (BSA) were not included in the study as these data were not available for the entire group of 50 patients comprising the study cohort. However, height, bodyweight and BSA have shown variable contribution to the warfarin dosing algorithm, though these parameters did not prove to have significant contribution to warfarin dosing in previous studies that recruited an Egyptian population. Shahin et al. [26] concluded that BSA, height and bodyweight were not significant predictors of dose. They reported that it might be possible that this was because of the minimal variation in height, which varied in the population by less than 4 %. Furthermore, El Din et al. [27] reported that bodyweight showed no influence on dose variability. Moreover, the target INR in our study was 2–3. In other studies, the contribution of target INR, which was between 2 and 3.5 or 2 and 4, might have contributed to the percentage of variation. Also, this study had a relatively small number of participants, whereas studying a larger number may have contributed to a higher percentage of variation. Finally, variation could be attributed to co-morbidity, concurrent medication and low albumin levels, which were not included in the algorithm.

In Egypt, warfarin is the most widely prescribed anticoagulant for reducing thromboembolic events because of its proven efficacy and incomparable low cost compared with the new generation of anticoagulants such as thrombin inhibitor and activated factor X (FXa) inhibitors that have been developed and are available on the international market [29].

To our knowledge, there were no warfarin pharmacogenomic studies carried in Egypt, with the exception of only three studies [26, 27, 30] in the preceding 2 years. The novel attribute of our study was the development of a warfarin dosing algorithm based on the genetic and nongenetic factors of the study cohort, and the validation of the calculated warfarin dosing algorithm in a different cohort with a significant positive correlation observed between the predicted warfarin dose and the actual prescribed dose. In an earlier study, in which 195 Egyptian patients were enrolled, Shahin et al. [26] reported that genetic polymorphisms in *VKORC1* 3673G>A, *CYP2C9* and *APOE*, along with nongenetic factors (age, smoking and pulmonary embolism), were determinants of warfarin dose requirements. El Din et al. [27] performed *VKORC1* 1173C>T and *CYP2C9**1,*2,*3 genotyping in 46 Egyptian patients and their regression analysis explained about 61 % of the variation in stable dose. Their study design didn't include validation of their regression model in a different cohort. Their regression model differed from ours in that it did not include the age variable and included the three variants of *CYP2C9* (*1*2, *1*3 and *2*2). The recent study carried by Bazan et al. [30] compared the performance of two published clinical and pharmacogenetic algorithms-Gage [18] and IWPC [19]-as well as the warfarin dosing table comprising *VKORC1* −1639G>A and *CYP2C9* genotypes with empiric dosing in a dataset of 63 Egyptian patients.

Our results show that the maintenance dose decreases, on average, by 0.4 mg/week for every 1 year increase in age. Gage et al. [31] reported a decrease in dose requirements with age, owing to reduced clearance and/or increased responsiveness, by 8–10 % per decade of life. Increasing age of patients is associated with a higher sensitivity to warfarin, which may be caused by the fall in total

hepatic content of VKOR because of the age-related decrease in hepatic mass [32]. Multiple linear regression models used to develop warfarin dosing algorithms have consistently found age to be a significant contributor to variability in dose requirements [7].

In accordance with the findings of this study, which show that gender did not contribute significantly to the observed variability in dose requirement, El Din et al. [27] and Teh et al. [33] concluded the same results. However, Cini et al. [34] and Dan et al. [35] found an association of male gender with higher warfarin requirements.

Previous studies [36, 37] concluded contradictory results regarding the influence of dietary factors such as alcohol consumption or vitamin K intake on warfarin dose requirements. Poor compliance with prescribed warfarin therapy may also cause either excessive bleeding or thrombosis response [37]. As all of the patients who were treated with warfarin in this study had received counselling, were put on stable diet plan and were monitored at least once monthly, it is unlikely that vitamin K had a significant impact on the warfarin dose requirements. To investigate the association between dietary factors and warfarin dosage, more controlled studies are needed to achieve reliable results. Among the different factors involved in dose variability are smoking, various illnesses and concurrent medications that were controlled in this study as part of the exclusion criteria.

As regards the frequency of *VKORC1* (1173C>T) and *CYP2C9* (1075A>C) gene polymorphisms, our results are similar to those of studies previously carried out on an Egyptian population [27, 30], while they were different from other ethnic groups as discussed in El Din et al. [27]. In the present study, the distribution of *VKORC1* (1173C>T) show deviation from Hardy Weinberg Equilibrium, which may be explained by the high inbreeding, with an average inbreeding coefficient of 0.0145 in the Egyptian population, as previously reported [38].

In the current study, an association between the *VKORC1* (1173C>T) SNP and the weekly warfarin maintenance dose was found. These results are consistent with other studies [27, 28]. The 1173C>T polymorphism in the *VKORC1* is a nucleotide substitution in intron 1 of the gene. Presence of the variant T nucleotide is associated with a lower warfarin daily dose requirement, and the 1173 SNP might be in linkage disequilibrium (LD) with other variants that alter *VKORC1* activity [28]. It is suggested that −1639A>G could be that variant. The −1639 promoter SNP is located in an E-Box in the 5′-untranslated region of the gene. The consensus sequence of this E-box is CANNTG. Changing the second base A to G, as observed in the −1639 site, would abolish the E-box consensus and would increase the promoter activity by 44 %. This suggested that E-box could function as a repressor binding site that represses transcription [39]. Moreover, a *VKORC1* −1639 G>A SNP qualitatively changed the expression of the VKORC1 protein [40].

We did not observe significant differences in warfarin dose demands between *CYP2C9**1*1 and *CYP2C9**1*3. Our findings are in concordance with previous studies carried on a Japanese [14], Chinese [41] and Indonesian population [23]. This is in contrast to other reports [7–9]. This discordance might be due to the following causes: (1) intake of concomitant drugs or diets that might have overruled the reduced activity of *CYP2C9* caused by the *3 allele; (2) the allele frequency of *CYP2C9**3 affecting the enzyme activity was too low to allow detection of a significant difference in our study cohort; (3) a selection bias due to a comparatively small sample of a strictly defined group of warfarin-treated patients, who were selected based on their very stable INR values; and (4) the influence of additional genetic factors that we did not account for, including multidrug resistance 1 (*MDR1*) [42] genes encoding vitamin K-dependent clotting factors [43], GGCX encoding γ-glutamyl carboxylase in the vitamin K cycle [44], the γ-glutamyl carboxylase inhibitory protein calumenin [45], apolipoprotein E [26] and possible genes encoding additional components of the VKOR complex [7].

There is an international debate on the use of pharmacogenetic information in guiding warfarin treatment [46, 47] and the cost effectiveness of analyses on genotype-guided dosing has been reported [48, 49]. As technology has improved and costs have decreased, the increasing availability of genomic testing promises genotype-tailored medical care [50]. In the present study, a two-tube assay was used for the simultaneous genotyping of *VKORC1* and *CYP2C9* polymorphisms separately under identical thermocycling conditions based on T_m-shift technology where the selected primers for both *VKORC1* and *CYP2C9* have a calculated T_m of 55–60 °C (excluding GC tail) [16]. Compared to other current methods [51] for genotyping *VKORC1* and *CYP2C9* genes, T_m-shift genotyping is simple and inexpensive. Primers are standard DNA oligonucleotides, to which generic SYBR Green fluorescent dye is added. Both alleles can be discriminated in a single closed-tube reaction simultaneously and results are obtained immediately after PCR. No post-PCR processing, such as enzymatic digestion, hybridisation or gel electrophoresis, is needed, which can eliminate one of the major causes of contamination in diagnostic PCR laboratories [16]. Although convenient allele discrimination by PCR using allele-specific TaqMan probes permits analysis of both alleles simultaneously, the cost of the probes should be taken into consideration [16]. Therefore, this technique is technically feasible for *VKORC1* and *CYP2C9* genotyping before beginning warfarin therapy.

This allows a clinician to prescribe the first warfarin dose within the same day [16].

The study is, however, not without weaknesses. First, the relatively small sample size, which may not have detected other determinant factors of warfarin dose or may have produced a less precise estimate for the regression coefficients, with the consequence that the new elaborated dosing algorithm included only factors with a high prevalence or a strong effect on warfarin dose. Another possible consequence of the relatively small sample size was the absence of the *CYP2C9**3*3 genotype in the study cohort and its presence in the validation group, though there was neither selection bias nor misclassification because patients were selected blindly of their genotype. In any case, the *CYP2C9**3 variant didn't contribute to the warfarin dose, so it was not included in our proposed dosing algorithm. Second, this study does not address whether a pharmacogenetically predicted dose of warfarin translates into better clinical endpoints, such as a reduction in the time to dose stabilization, fewer out-of-range INRs and/or a reduced incidence of bleeding episodes.

5 Conclusion

In Egyptian patients, *VKORC1* (1173C>T) contributes to warfarin dose variability. Patients' age and genetic variants of *VKORC1* account for nearly 20.5 % of the variability in warfarin dose required to achieve an INR of 2–3. On validation of the suggested warfarin dosing algorithm in a different cohort, the predicted dose correlated significantly, with sufficient strength, with the maintenance dose and the equation was more successful among patients with a dose ≥35 mg/week; out of 16 patients with a dose ≥35 mg/week, 11 (68.8 %) showed dose agreement.

It appears to be appropriate to increase the number of candidate genes to include those such as *CYP4F2*, which has a moderately significant effect on warfarin dose as previously reported [52], and those involved in the metabolism of warfarin to set up a powerful tool that is easy for rapid use in all laboratories and clinical settings, to improve warfarin therapy management. In addition, differences in ethnic backgrounds should be taken into account in models producing quantitative dosing algorithms. Replication of this study in a larger cohort of patients is necessary before translating this knowledge into clinical guidelines for warfarin prescription. This would likely have a major impact on patient safety and the efficacy of warfarin during both the initiation and maintenance of therapy, reducing the burden of frequent INR measurements and improving safety by reducing the risk of over- or under-anticoagulation in Egyptian patients.

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