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Enzyme Polymorphism in Warfarin Dose Management After Pediatric Cardiac Surgery

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Background: Warfarin is an anticoagulant and is widely used for the prevention of thromboembolic events. Genetic variants of the enzymes that metabolize warfarin, i.e. cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1), contribute to differences in patients' responses to various warfarin doses. There is, however, a dearth of data on the role of these variants during initial anticoagulation in pediatric patients.

Objectives: We aimed to evaluate the role of genetic variants of warfarin metabolizing enzymes in anticoagulation in a pediatric population.

Patients and Methods: In this prospective cohort study, 200 pediatric patients, who required warfarin therapy after cardiac surgery, were enrolled and divided into two groups. For 50 cases, warfarin was prescribed based on their genotyping (group 1) and for the remaining 150 cases, warfarin was prescribed based on our institute routine warfarin dosing (group 2). The study endpoints were comprised of time to reach the first therapeutic international normalization ratio (INR), time to reach a stable warfarin maintenance dose, time with over-anticoagulation, bleeding episodes, hospital stay days and stable warfarin maintenance dose.

Results: There was no significant difference concerning the demographic data between the two groups. The time to stable warfarin maintenance dose and hospital stay days were significantly lower in group 1 ($P < 0.001$). However, there was no statistically significant difference in time to reach the first therapeutic INR, time with over-anticoagulation and bleeding episodes, between the two groups.

Conclusions: The determination of warfarin dose, based on genotyping, might reduce the time to achieve stable anticoagulation of warfarin dose and length of hospital stay.

Keywords: Anticoagulants; Genotype; Pediatrics; Warfarin

1. Background

Oral anticoagulation with vitamin K antagonist, warfarin, is the most frequently prescribed oral anticoagulant worldwide (1). Nonetheless, warfarin anticoagulant therapy is difficult to manage due to the narrow therapeutic index and wide variability of dose response among individuals (1-3). Warfarin doses are adjusted to maintain each patient's international normalized ratio (INR) within a narrow therapeutic range. Environmental factors such as age, body weight, diet, and concomitant medication, as well as genetic factors, such as cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) are well known to affect warfarin dose requirement (1, 4-29).

Warfarin is administered as a racemic mixture of (R)- and (S)-warfarin, of which the more potent (S)-warfarin is metabolized by CYP2C9. (10) Polymorphisms in the gene encoding CYP2C9 are recognized to contribute to variability in sensitivity to Warfarin (8, 9). Patients with significant differences in *CYP2C9* genotype may require a lower dose of warfarin and a longer time to reach a stable dose. This group of patients is also at risk for over-anticoagulation and serious bleeding events (1, 3, 22).

The VKORC1 reforms vitamin K epoxide to a reduced form of vitamin K, which is a required cofactor for the activation of clotting factors II, VII, IX, and X and proteins C, S, and Z, by gamma-glutamyl carboxylation (6, 7). Polymorphisms in the gene coding VKORC1 are associated with a lower warfarin dose requirement (6, 7, 23, 28). In several studies, *VKORC1* polymorphisms were found to contribute to the differences in dose requirement, more than *CYP2C9* variants (5, 10, 15, 25, 27).

Considering the fact that a safe and effective dose, for an individual patient, is determined empirically, the first months of warfarin treatment are problematic. As a result, the risk of over-anticoagulation and hemorrhagic events is higher during this time (2, 11).

Genotyping is, therefore, likely to have the most significant effect when individualizing the warfarin dose, during the initiation of treatment (15). Nevertheless, the existing literature contains only a few studies on the relative contributions of *VKORC1* and *CYP2C9* to the anticoagulation response in pediatric patients during the initiation of warfarin therapy (4-6).

2. Objectives

Therefore, we sought to evaluate the effect of variant *CYP2C9* and *VKORC1* alleles to determine the warfarin dose requirement in pediatric patients during the early phase of anticoagulant treatment.

3. Patients and Methods

3.1. Patient Selection

A total of 200 consecutive pediatric patients, who required anticoagulant therapy after cardiac surgery, between September 2010 and March 2012, were enrolled. The exclusion criteria comprised underlying conditions that influence drug dosage, such as cancer, renal or hepatic insufficiency, and congestive heart failure or consumption of a drug that might potentiate or attenuate the anticoagulant effect of warfarin.

The study was reviewed and approved by the research deputy and local ethics committee and a written informed consent was obtained from all the patients' parents.

Primary evaluation and clinical history were obtained from all patients, and detail demographic, concurrent medications, cardiac diagnoses, and types of cardiac surgery were recorded .The study population, thereafter, were divided into two groups, according to their consent for genotyping: group 1 included 50 cases in whom warfarin was prescribed, based on genotyping, and group 2 included 150 cases, in whom warfarin was prescribed, based on the institute routine protocol (considering the number of 50 for cases whose genotyping was performed for, while 150 patients were randomly chosen, as the control group, among pediatric patients who required warfarin after cardiac surgery). For all patients in the study, warfarin therapy was initiated at the second day after surgery and the target INRs were considered based on recommended guidelines for children (2, 11).

3.2. Warfarin Dosing in the Group 1 (Genotyping Method)

For patients in group 1, 5 - 10 mL venous blood sample was obtained and referred to molecular genetic laboratory for genotyping. In the first step of the procedure, DNA extraction was performed with Genomic DNA purification kit (Lot: 00145461, Fermentas Co., Vilnius, Lithuania). The DNA concentration and quality were evaluated by BioPhotometer. Samples with 1.7 - 2.0 purity (OD = 100 ng) were applied for the study. The extracted DNA were then genotyped for two genes, *VKORC1* and *CYP2C9*, using a Food and Drug Administration (FDA)-approved kit (Lot: 31045, Aid Diagnostika GmbH, Strasberg, Germany) by polymerase chain reaction (PCR) reverse dot blots. Suspicious cases were approved by PCR- restriction fragment length polymorphism (RFLP) method.

The results of genotyping reported *VKORC1* normal (*G*) and mutant type (*A*) and *CYP2C9* normal (wild) type (**1*) and its **2* and **3* polymorphisms.

Sensitivity to warfarin and recommended dose, based on the genotyping results, were reported by genetic laboratory, considering the international warfarin pharmacogenetics consortium algorithms (Tables 1 and 2) (30). Thereafter, the required dose of warfarin in each pediatric patient was determined, considering the dose recommended by genotyping and adjusted according to the patient's height, weight, and body surface area, using the Pennas, Salisbary, and Clarks formula (31).

Table 1. Sensitivity to Warfarin Based on Combined *CYP2C9* and *VKORC1* Genotyping [a]

Warfarin Sensitivity	*CYP2C9* Genotype	*VKORC1* Genotype
Normal	**1/*1*	*G/A*
Less than normal	**1/*1*	*G/G*
Mild	**1/*2*	*G/G*
	**2/*2*	*G/G*
	**1/*3*	*G/G*
Moderate	**2/*3*	*G/G*
	**1/*2*	*G/A*
	**2/*2*	*G/A*
	**1/*3*	*G/A*
	**1/*1*	*A/A*
High	**3/*3*	*G/G*
	**2/*2*	*G/A*
	**1/*2*	*A/A*
Very high	**3/*3*	*G/A*
	**2/*2*	*A/A*
	**1/*2*	*A/A*
	**2/*3*	*A/A*
	**3/*3*	*A/A*

[a] By permission of Peyvandlab, Tehran, Iran.

Table 2. Initial Recommended Warfarin Doses (mg/day), Based on Genotype

CYP2C9 Variants	*VKORC1 GG* Initial Dose	*VKORC1 AG* Initial Dose	*VKORC1 AA* Initial Dose
None	5.6	4.5	3.5
CYP2C9*1/*2	4.5	3.5	2.7
CYP2C9*1/*3	4.0	3.1	2.3
CYP2C9*2/*2	3.5	2.7	2.0
CYP2C9*2/*2	3.1	2.3	1.6
CYP2C9*3/*3	2.6	1.9	1.3

3.3. Warfarin Dosing in the Group 2 (Conventional Method)

For group 2, based on our approved institutional protocol and by daily assessment of prothrombin time (PT) and INR, warfarin dose was estimated to be 0.2 mg/kg. The dose of warfarin was increased or decreased by 10% - 15% until proper anticoagulative state was achieved.

After discharge, all patients were subsequently followed for 3 months after initiation of warfarin and their anticoagulation status and possible complications were recorded.

3.4. Study Endpoints

The study endpoints included target INR, genetically predicted warfarin dose, calculated warfarin daily dose, stable warfarin maintenance dose (SAD), time to target INR, time to stable anticoagulation, time with over anticoagulation, hospital stay days and bleeding episodes. Table 3 depicts the definitions of study endpoint variables.

3.5. Statistical Analysis

The IBM SPSS Statistics 19.0 for Windows (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. All data were initially analyzed, using the Kolmogorov-Smirnov test, to assess for normal distribution. Categorical variables are presented as numbers and percentages and quantitative variables as mean and standard deviation. Categorical data were compared by Chi-square test, while quantitative variables by Student t-test or Mann-Whitney test, as appropriate. For comparative analysis between SAD and different genotypes of *VKORC1* and *CYP2C9*, one way analysis of variance (ANOVA) was used. The regression model was used to assess the correlation between the SAD and genetically predicted warfarin dose. A $P < 0.05$ was considered statistically significant.

4. Results

The study population comprised of 200 patients, who required warfarin treatment for the first time, due to valve replacement or single ventricular approaches, such as Glenn or Fontan operation. Totally, 41 patients in group 1 and 118 patients in group 2 had a history of valve replacement, whereas the rest of them had single ventricular approaches.

Table 4 depicts demographic characteristic of the study population. There were no statistical significant differences between the two groups, regarding the mean of age, weight, height and body surface are (BSA). A proportion of 70% of patients in group 1 and about 61% in group 2 were male.

Table 3. Definitions for Study Variables and Endpoints

Variable	Definition
Target INR	Recommended therapeutic INR range based on specific diagnoses. The target INR was considered 2 for single ventricular approaches and 2.5 for valve replacement operations
Genetically predicted warfarin dose	Recommended warfarin dose based on genotyping tests
Calculated warfarin daily dose	Adjusted recommended warfarin dose based on the body surface area, Clarcks, Salisbary, and Pennas formulations in pediatric patients.
Stable warfarin maintenance dose	The warfarin dose, when three consecutive INR measurements in 3 weeks were within a therapeutic range, from the same mean daily dose
Time to target INR	Time to reach first therapeutic INR
Time to stable anticoagulation	Time to reach a stable warfarin maintenance dose
Time with over anticoagulation	Time with an INR > 4, as an index for the risk of over-anticoagulation
Bleeding episodes	At least one episode of significant mucosal, urinary or gastrointestinal bleedings requiring urgent intervention as anticoagulation reversal or transfusion
Thrombotic events	Any thrombotic event, including prostheses thrombosis and/or any embolic event after surgery

Table 4. Demographic Data of Study Population [a,b,c]

	Group 1 (n = 50)	Group 2 (n = 150)	P Value
Age, y	11.4 ± 3.4 (5 - 17)	11 ± 3.3 (3.5 - 16)	0.5
Gender			0.5
Female	15 (30)	59 (39.3)	
Male	35 (70)	91 (60.7)	
Height, cm	138.5 ± 18.1 (102 - 171)	134 ± 17.7 (94 -170)	0.5
Weight, Kg	36.8 ± 14.5 (13 - 65)	34.9 ± 13.8 (12 - 75)	0.1
BSA, m^2	1.17 ± 0.3 (0.6 - 1.7)	1.12 ± 0.3 (0.6 - 1.8)	0.3

[a] Abbreviation: BSA; body surface area.
[b] Data are described as No. (%) for qualitative variables and as mean ± SD (range) for quantitative variables.
[c] Group 1, patients with pharmacogenetic testing; and group 2, patients without pharmacogenetic testing.

4.1. Genotyping Frequencies

In the 50 pediatric patients who were investigated for polymorphism in *VKORC1* and *CYP2C9*, the frequency of normal (wild type) genome for *VKORC1* and *CYP2C9* were found to be 44% and 48%, respectively. The frequency for *CYP2C9* different variants was 56% and for *VKORC1* was 52%. Table 5 depicts the frequencies of different alleles, in detail. The sensitivity to warfarin based on combined *CYP2C9* and *VKORC1* genotyping in this group are shown in Table 6. As shown in this table, moderate sensitivity to warfarin is more prevalent (40%) in this study population, which could be due to more prevalent mutant alleles responsible for moderate sensitivity to warfarin of both *VKORC1* and *CYP2C9* genotypes (Tables 1 and 5).

4.2. Determination of Warfarin Dose by Genotyping

Based on recommended dose in laboratory results, the adjusted dose for pediatric patients was calculated, as explained in the methods section. In Table 7, the recommended, adjusted and SAD, i.e. warfarin dose when three consecutive INR measurements were within a therapeutic range from the same mean daily dose, have been shown. As shown in this table, the stable SAD is very similar to the adjusted dose among different types of alleles. However, SAD for patients with genotype of *1*1*/AA* is considerably higher than the recommended and adjusted dose (5.6 versus 3.3 mg/day).

The comparison of SAD between different genotypes of *VKORC1* and *CYP2C9* showed that SAD seems to be influenced by both *VKORC1* and *CYP2C9* genotypes (Table 8). As shown in Table 8, SAD is significantly higher in *1*1** genotype for *CYP2C9* gene (P = 0.014), whereas for *VKORC1* gene, SAD for *GG* genotype was significantly higher than the *GA* genotype (P <0.002). Moreover, SAD for *AA* genotype seems to be higher than the other genotypes. However, regarding the small number of patients (only two patients), it cannot be conclusive.

The Spearman correlation analysis showed a moderate, although significant, association between SAD and genetically predicted warfarin dose (r = 0.5, P <0.001)

4.3. Comparing Endpoint Variables Between Groups 1 and 2

The time to stable anticoagulation and hospital stay days were significantly lower in patients with genetically adjusted warfarin dose (group 1, P <0.001). However, there was no statistically significant difference in time to reach the first therapeutic INR and time with over anticoagulation between two groups (Table 9).

There were only six significant bleeding episodes among our study population; two episodes in group 1 and four episodes in group 2. Because of the small number of bleeding episodes, statistical analysis does not seem to be rational. There was also no thrombotic event in our study population, during hospital stay or follow up.

Table 5. Genotype Frequencies in Group 1 [a,b,c]

VKORC1[b]	*CYP2C9*[c]						Total
	*1*1*	*1*2*	*1*3*	*2*2*	*2*3*	*3*3*	
GG	11 (22)	2 (4)	1 (2)	6 (12)	4 (8)	0	24 (48)
GA	9 (18)	4 (8)	1 (2)	9 (18)	1 (2)	0	24 (48)
AA	2 (4)	0	0	0	0	0	2 (4)
Total	22 (44)	6 (12)	2 (4)	15 (30)	5 (10)	0	50 (100)

[a] Data are described as No. (%).
[b] Genotype variants for vitamin K epoxide reductase gene.
[c] Genotype variants for cytochrome P450 2C9.

Table 6. Sensitivity to Warfarin, Based on Combined *CYP2C9* and *VKORC1* Genotyping in Our Study Population (Group 1) [a]

Sensitivity	Values
Less than normal	11 (22)
Normal	9 (18)
Mild	9 (18)
Moderate	20 (40)
High	1 (2)

[a] Data are described as No. (%).

Table 7. Initial Warfarin Doses (mg/day) Recommended, Based on Genotype, Adjusted for Pediatrics and Stable Anticoagulation Dose in our Study Population (Group 1) [a,b]

VKORC1	*CYP2C9*														
	*1*1**			*1*2**			*1*3**			*2*2**			*2*3**		
	R	A	SAD	R	A	SAD	R	A	SAD	R	A	SAD	R	A	SAD
GG	5.6 [b]	4.1 ± 1.2	4.5 ± 1.6	4.5	4	3.9 ± 0.2	4	3.1	3	3.5	3.2 ± 0.9	3.9 ± 1.6	3.1	2.4 ± 0.3	2.5 ± 0.5
GA	4.5	3.1 ± 0.9	3.5 ± 1.3	3.5	1.97 ± 0.8	1.96 ± 0.7	3.1	2.3	2.5	2.7	2.7 ± 0.5	2.7 ± 0.5	2.3	1.6	1.8
AA	3.5	3.3 ± 0.2	5.6 ± 0.9	-	-	-	-	-	-	-	-	-	-	-	-

[a] A, Adjusted dose; R, Recommended dose; SAD, stable anticoagulation dose.
[b] mg/day.

Table 8. Stable Warfarin Maintenance Dose in Different Genotypes of *VKORC1* and *CYP2C9* [a,b,c]

	Values	SAD	P Value
CYP2C9 [b]			0.014
*1*1**	22 (44)	4.2 ± 1.6	
*1*2**	6 (12)	2.6 ± 1.2	
*1*3**	2 (4)	2.7 ± 0.3	
*2*2**	15 (30)	3.2 ± 1.2	
*2*3**	5 (10)	2.4 ± 0.6	
VKORC1 [c]			0.002
GG	24 (48)	3.9 ± 1.5	
GA	24 (48)	2.4 ± 1.1	
AA	2 (4)	5.6 ± 0.9	

[a] Data are presented as No. (%) or Mean ± SD.
[b] Genotype variants for vitamin K epoxide reductase gene.
[c] Genotype variants for cytochrome P450 2C9.

Table 9. Comparing Endpoint Variables Between Group 1 and 2 [a,b]

	Group 1 (n = 50)	Group 2 (n = 150)	P Value
Time to target INR, day	3.4 ± 1.2	3.5 ± 1.4	0.6
Time with over anticoagulation, day	2.9 ± 0.8	3.1 ± 0.8	0.09
Hospital stay days	22.6 ± 4.2	30.2 ± 6	< 0.001
Time to stable anticoagulation, day	32.8 ± 6	41.5 ± 6.5	< 0.001

[a] Data are presented as mean ± SD.
[b] Group 1, patients with pharmacogenetic testing; group 2, patients without pharmacogenetic testing.

5. Discussion

Warfarin is a drug of choice after valve replacement surgery and single ventricular approaches and every surgical modality, with thromboembolic tendency (4-6). In this study, we evaluated the distribution of two genes involved in sensitivity to warfarin (*VKORC1* and *CYP2C9*) in children and the effect of a pharmacogenetic-based dosing model in anticoagulation status and complications among them.

The current study showed that the distribution of normal (wild) genome for both *VKORC1* and *CYP2C9* genes are less frequent than their variant alleles (44% and 48%, respectively). These results are somehow different from the results of similar studies (5, 6). Nguyen et al. (5) showed a frequency of 73% for *CYP2C9* wild genome and 27% for *VKORC1* wild genome. About 36% of children in Biss et al. (4) study had a genotype of *GG* for *VKORC1* and 70% showed wild type (*1*1**) genotype for *CYP2C9* gene. In the studies of Wadelius et al. (24) and Wen et al. (25), *CYP2C9* variants were detected prominently less than *VKORC1* variants in an Asian population. However, in our study, the variant alleles of *CYP2C9* and *VKORC1* were seen in 56% and 52% of the pediatric patients, respectively. It is better to clarify

in another study whether these differences are due to racial differences or are because of the small sample size of the current study. Nguyen et al. (5) demonstrated that the strongest influence on daily warfarin dose in pediatric population exerted by the *VKORC1* genotypes and the *CYP2C9* genotypes accounted for only 5% of total dosing variability. This study showed that, among our study population, both *VKORC1* and *CYP2C9* genes have influence on SAD (Table 6) and we could not conclude which gene would have more effect on warfarin dose variability.

Warfarin has a narrow therapeutic window that requires careful clinical management to balance the risks of over-anticoagulation and bleeding, with those of under-anticoagulation and clotting (20). The reliable international survey by Takahashi et al. (12) demonstrated that genotyping, accompanied by the demographic criterion, could predict up to 64% of warfarin dose differences, in adults. In multiple studies, allele frequencies in the two different genders were examined and finally, the FDA-approved genetic-based warfarin dose prediction model was published (30). Nguyen et al. (5) investigated the clinical and genetic determinants of warfarin dose adjustment in children who require warfarin for their cardiovascular problems and suggested that genetic polymorphisms together with clinical factors would be important determinants for warfarin dosing in children.

In this study, we used a FDA-predicted dose model for warfarin, at first, and thereafter adjusted the recommended dose in each child, based on the body surface area. Clarcks, Salisbary, and Pennas formulations (31) showed a moderate association between the predicted warfarin dose, based on genotyping, and the stable anticoagulation dose ($r = 0.5$, $P < 0.001$). The other studies have demonstrated similar results in their studies (18, 21, 22). However, Biss et al. (4) concluded that warfarin dose was overestimated in their cohort of pediatric population, by using the pharmacogenetic-based warfarin dosing algorithm, published by The International Warfarin Pharmacogenetics Consortium.

We demonstrated that stable anticoagulation dose achievement and hospital discharge were accomplished 1 week earlier, in group 1, and, by using the pharmacogenetic-based dosing model, the time to stable anticoagulation and hospital stay days were significantly decreased. A shorter length of hospital stay may reduce the risk of delay in achieving therapeutic INR and would be cost effective. However, the cost effectiveness of this method should be addressed in another study.

Wen et al. (25) and Veenstra et al. (26) demonstrated the minimum genetically predicted warfarin doses of 1.8 - 1.9 mg/day. More than 80% of the patients achieved therapeutic INR and stable anticoagulation dose within 2 - 6 weeks. Mutant genotypes resulted from these entities within 1 - 2 weeks later (24, 27). Our results were similar to their studies, except for the fact that there was no significant difference in achieving therapeutic INR and SAD between the various genotypes in our study.

5.1. Study Limitations

The most important study limitation was the relatively small sample size of the study and, because of the limited number of patients who had genetic variants, several analyses would not be conclusive enough. The lack of data about genetic polymorphisms of *CYP2C9* and *VKORC1* in our control group was another limitation of this study.

The results of our study suggest that adjustment of warfarin dose by *CYP2C9* and *VKORC1* genotyping could be associated with earlier stable warfarin dosing and decreased length of hospital stay, in pediatric population. However, the observed differences in genes distribution in our study population should be addressed in another study. It is recommended to design similar studies, with reasonable sample volume, for better defining the prevalence of *VKORC1* and *CYP2C9* variants and for developing a warfarin dosing algorithm in pediatric population.

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Authors' Contributions

Avisa Tabib: Conception and designing the study, collecting data, analysis and interpretation of data, drafting of the manuscript; Babak Najibi: Collecting data, interpretation of data and drafting of the manuscript; Mohammad Dalili: Interpretation of data and revising of the manuscript; Ramin Baghaei: Interpretation of data and revising of the manuscript; Behzad Poopak: Drafting and revising the manuscript.

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