Research Article

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Pharmacogenomics

Pharmacogenetic variants influence vitamin K anticoagulant dosing in patients with mechanical prosthetic heart valves

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Background: Vitamin K antagonists (VKAs) are class I oral anticoagulants that are widely prescribed following surgical heart valve implantation. The objective of this study was to quantify the relative effects of *VKORC1, CYP2C9* and *CYP4F2* genotypes in predicting VKA dosing. **Materials & methods:** A total of 506 South Indian patients with mechanical prosthetic heart valves who were prescribed oral VKAs, such as warfarin or acenocoumarol, were genotyped. The discriminatory ability of mutant genotypes to predict dose categories and bleeding events was assessed using regression analysis. **Results:** The *VKORC1* rs9923231, *CYP2C9*3* and *CYP4F2*3* mutant genotypes significantly influenced VKA-dose requirements and explained 27.47% of the observed dose variation. **Conclusion:** These results support pharmacogenetic screening for initial VKA dosing among South Indian patients with mechanical prosthetic heart valves.

First draft submitted: 29 January 2022; Accepted for publication: 6 May 2022; Published online: 24 May 2022

Keywords: acenocoumarol • antagonists • anticoagulant • genotype • heart valve • pharmacogenomics • regression • vitamin K • warfarin

Background

Vitamin K antagonists (VKAs) are widely prescribed in patients who require prophylactic and therapeutic anticoagulation following events such as venous thromboembolism, mechanical heart valve implantation and atrial fibrillation [1]. These inhibitors of vitamin K epoxy reductase deplete the active form of vitamin K and act indirectly on the process of glutamate carboxylation of clotting factors in the liver [2–4]. However, warfarin, and other clinically used VKAs such as acenocoumarol and phenindione, have long half-lives and narrow therapeutic indices that make dosing an iterative process extending from weeks to months [5]. Therefore, to maintain a stable dose, regular monitoring of prothrombin time/international normalized ratio (PT/INR) is essential. The failure to do so exposes the patient to risks of bleeding or thromboembolism in the event of over- or undercoagulation, respectively. Despite efforts to maintain regular monitoring, wide interindividual variability in dosing has been observed among patients with the same target PT/INR, which has been attributed to the presence of genetic variants in drug-target and drug-metabolizing genes [6].

Population-based association studies have reiterated the need for regenotyping genetic variants in pharmacogenes to confirm the utility of pharmacogenetics-guided dose determination in specific populations [7]. Genotype-based dosing was shown to be superior compared with the standard management based on evidence from the Genetics-

InFormatics randomized controlled trial (GIFT) [8]. The outcomes of the GIFT trial indicated that screening of *CYP2C9*, *VKORC1* and *CYP4F2* genes improved the safety of warfarin initiation among patients who underwent elective hip or knee arthroplasty to prevent deep vein thrombosis. The duration of time that the patient remained in the target therapeutic range (TTR) was increased in those who received genotype-guided dosing. Such patients experienced a lower risk of death or complications due to major bleeding events and venous thromboembolism compared with those who received standard management [8]. Following the publication of the results of the GIFT trial, updated guidelines for the genotype-based dosing of warfarin were derived by the Clinical Pharmacogenetics Implementation Consortium (CPIC) using the data reported from individuals of African American, East Asian and European ancestry [6]. Notably, the CPIC guidelines recommend a genotype panel containing variants in *VKORC1*, *CYP2C9* and *CYP4F2* genes for conducting pharmacogenetic dosing for warfarin.

However, similar data from randomized controlled trials that could provide information regarding the utility of genotype-guided dosing is lacking from South Asians, who constitute a majority of the world population. Genetic mapping of patient samples from diverse populations has revealed that the allelic diversity underlying disease is predictable and can be utilized for mapping pharmacogenomic traits [9]. In the present study, the mean VKA-dose requirements were compared among different genotype carriers and the proportional odds and marginal effects of pharmacogenetic variants in low- to intermediate- or high-dose categorizations were estimated. The differences in the variation explained by the non-genetic versus genetic factors for VKA dose weekly requirement were compared and the risk of VKA-induced bleeding among patients implanted with prosthetic mechanical heart valves was also assessed.

Materials & methods

The participants included 506 patients recruited from the anticoagulation clinics of five participating centres in Kerala, India. Inclusion criteria were patients (≥18 years) who had undergone surgical mechanical heart valve replacement in mitral and/or aortic position, with normal prosthetic valve function and were on a stable maintenance dose of VKAs; that is, without any change in the mean daily dose of VKAs and maintaining a PT/INR between 2.5–3.5 over three visits spaced by a month between successive visits. The VKAs included warfarin or acenocoumarol; phenindione was not available on market. Exclusion criteria included patients with renal or hepatic dysfunction.

DNA isolation & Kompetitive Allele-Specific™ polymerase chain reaction genotyping

Isolation of genomic DNA was done using a rapid salting-out protocol from 3 ml of whole blood [10]. Biallelic discrimination of the *CYP2C9*2* rs1799853, *CYP2C9*3* rs1057910, *VKORC1* rs9923231 and *CYP4F2*3* rs2108622 variants was carried out using Kompetitive Allele-Specific PCR (KASP™) genotyping chemistry from LGC Genomics (Teddington, UK). Total reaction volumes of 10 µl were set up based on the manufacturer's protocol, using 10 ng genomic DNA, KASP Low ROX Master mix and KASP Assay mix that contained the target-specific primer (Supplementary Table 1). KASP genotyping involved two-step thermal cycling conditions with an initial activation step at 94°C for 15 min, followed by the first step, 10 cycles of denaturation at 94°C for 20 s and annealing/elongation at 61–55°C for 60 s with a ramp rate of 0.6°C per cycle. The second step included 26 cycles of denaturation and annealing/elongation at 94°C for 20 s and 55°C for 60 s, respectively. To improve genotype clustering, 3 additional cycles of denaturing and annealing/elongation were done at 94°C for 20 s and 57°C for 60 s, respectively. Amplification and plate reading were done on a QuantStudio 3 Real-Time PCR System (Applied Biosystems Inc., MA, USA). Allelic discrimination analysis and genotype scoring were done using QuantStudio-Design and Analysis Software v.1.4.3 (ABI, MA, USA). Representative genotypes of all four SNPs (single-nucleotide polymorphisms) were validated by Sanger sequencing with primers designed using Primer Premier software v5.0 (CA, USA) and used as positive controls in each plate run (Supplementary Table 2).

Statistical analysis

Data analysis was done using Stata v.16.0 statistical software (Stata Corp, TX, USA), and statistical significance was indicated by a p-value < 0.05. Hardy–Weinberg equilibrium (HWE) tests and allele frequency estimations were done using the genhw package. The VKA dose was calculated based on the transition factor of 1.85 mg between acenocoumarol and warfarin [11]. This value was square-root transformed to obtain the dependent variable having a normal distribution so as to satisfy the conditions for parametric testing. Normality testing was done using the Shapiro–Wilk test. The differences in mean daily dose requirements among heterozygote and

homozygote mutation carriers were calculated using the ANOVA test. Non-genetic independent variables, such as age, gender, height, weight, BMI, tobacco smoking, primary hypertension, diabetes mellitus, dyslipidemia, hypothyroidism, warfarin/acenocoumarol use, VKA indication and other prescribed medications, were assessed for selection into the genetic model using stepwise multiple regression. The non-genetic factors found to be statistically significant were incorporated into the genetic model. A multiple linear regression model adjusted for age, gender, warfarin/acenocoumarol use and angiotensin-converting enzyme (ACE) inhibitors was tested to detect the associations of the mutant genotypes with the square root of the VKA weekly average dose. The proportion of variance in the PT/INR-based VKA dose estimated by both the non-genetic and genetic models was measured by calculating R^2 and adjusted R^2.

Ordered logit regression analysis was used to assess the predictive ability of the genetic variants to allocate patients to different dose categories. The stable weekly anticoagulant dose was, therefore, categorized into low-dose (21 mg per week), high-dose (49 mg per week) and intermediate-dose (21–49 mg per week) groups, in accordance with previous guidelines [3]. These dose categories were used as the dependent variable in ordered logit regression analysis, adjusted for age and sex. Marginal effects of the genetic variants on mean VKA weekly dose were estimated using a multiple linear regression model using the *mfx* command. Gene × gene interactions were assessed by adding their multiplicative terms into models containing their marginal terms. The multiplicity-adjusted p-value was calculated using the Bonferroni method to control for multiple tests [12]. The association of mutant genotypes with the risk of VKA-induced bleeding events was analyzed using Fisher's exact test and logistic regression, adjusting for target PT/INR and anticoagulant indication, and all patients were included.

Results

A total of 506 patients were recruited into the study with a mean age of 50.44 ± 12.9 years. This comprised 243 (48.02%) female patients. The demographic characteristics, concomitant diseases, medications and VKA indications for the participants are summarized in Table 1. Other comorbidities such as primary hypertension, diabetes, dyslipidemia and hypothyroidism within this cohort were below 10%. The majority of the patients (48.8%) had mitral valve replacement (MVR) as the main indication for anticoagulants. Univariate analysis showed that age and male gender were negatively associated with weekly VKA dose (coef. = -0.015, p = 0.0004; coef. = -0.226, p = 0.039, respectively). The type of VKA used, either warfarin or acenocoumarol, and the use of ACE inhibitors were associated with higher weekly VKA dose requirements (coef. = 0.644, p = 0.0004; coef. = 0.371, p = 0.047, respectively). High-throughput SNP genotyping was done using the KASP assay and confirmed by Sanger sequencing. The frequencies of the genotypes and alleles of genetic variants are shown in Table 2. No significant deviation from HWE ($p > 0.05$) was observed in the sample. Among these polymorphisms, *CYP2C9*2* rs1799853 had the lowest minor allele frequency (MAF) of 3.5% in South Indian patients and the homozygous TT genotype was totally absent.

Association of genotypes with dose & dose categories

A significant difference in the mean PT/INR-based daily doses was observed among the genotype carriers of the *VKORC1* -1639G >A rs9923231, *CYP2C9*3* rs1057910 and *CYP4F2*3* rs2108622 polymorphisms ($p < 0.05$), as shown in Table 2. The proportional odds of a heterozygote or homozygote mutation carrier being assigned to the low-dose category versus the odds of being in the combined categories of intermediate- plus high-dose is also shown in Table 2. In the dominant genetic model, carriers of *VKORC1* rs9923231 and *CYP2C9*3* rs1057910 mutant genotypes had significantly higher adjusted odds of being in the low-dose category compared with the wild-type genotype carriers (ORs = 3.78 and 3.96, respectively; $p < 0.001$). On the contrary, carriers of the *CYP4F2*3* mutant genotypes had higher odds of being in the high-dose category (OR = 1.61; p = 0.017). No gene × gene interaction effects were found to influence the dose for the studied variants ($p < 0.05$). Figure 1 shows the crosstabulation of the mean PT/INR-based weekly dose in the sample for all genotype combinations of *CYP2C9-VKORC1-CYP4F2* genes, based on the Coriell Personalized Medicine Collaborative (CPMC) dosing guide [13].

Multiple linear regression analysis & vitamin K antagonist dosing algorithm

The stepwise multiple regression model identified four variables, age, male gender, warfarin/acenocoumarol use and ACE inhibitors, significantly associated with VKA dose. No association was observed for enzyme inducers such as phenytoin or with enzyme inhibitors such as amiodarone or statins. However, a significant interaction of *CYP2C9*2* CT genotype with ACE inhibitors was observed for VKA dose (coef. = 2.13; p = 0.039). Males required a

Table 1. Demographics, medications and indications for patients receiving vitamin K antagonists.

Characteristics	n = 506
Age in years, mean ± SD	50.42 ± 12.9
Female, n (%)	243 (48.02)
Height in cm, mean ± SD	159.32 ± 8.6
Weight in kg, mean ± SD	60.40 ± 10.87
BMI in kg/m^2, mean ± SD	23.74 ± 3.60
Comorbidities, n (%)	
Primary Hypertension	42 (8.30)
Diabetes	32 (6.32)
Dyslipidemia	44 (8.70)
Hypothyroidism	9 (1.78)
Medications, n (%)	
ACE inhibitors	48 (9.49)
ARBs	9 (1.78)
Calcium channel blocker	72 (14.23)
Beta blocker	170 (33.6)
PPIs	16 (3.16)
NSAIDs	144 (28.46)
Antibiotics	183 (36.17)
Diuretics	301 (59.49)
Thyroid hormone replacement	5 (0.99)
Aspirin/acetylsalicylic acid	126 (24.9)
Clopidogrel	41 (8.1)
Digoxin	185 (36.56)
Statins	90 (17.79)
Amiodarone	20 (3.95)
Eptoin	3 (0.59)
Anticoagulant indication, n (%)	
AF	37 (7.3)
AVR	150 (29.6)
BMV	20 (4.0)
CMV	13 (2.6)
DVR	38 (7.5)
MVR	247 (48.8)
TVR	1 (0.2)

ACE: Angiotensin-converting enzyme; ARBs: Angiotensin receptor blockers; AF: Atrial fibrillation; AVR: Aortic valve replacement; BMV: Balloon mitral valvotomy; CMV: Closed mitral valvotomy; DVR: Double valve replacement; MVR: Mitral valve replacement; NSAIDs: Nonsteroidal anti-inflammatory drugs; PPIs: Proton pump inhibitors; TVR: Triple valve replacement.

relatively lower VKA dose than female patients (coef. = -0.28; p = 0.0035). The non-genetic factors explained 9.8% of the weekly average VKA dose. When mutant genotypes were added, the model was able to explain an additional 17.67% of the observed VKA-dose variation. In total, the full genetic model explained 27.47% of the dose variation in the sample (Table 3). Based on the estimated coefficients of regression, the regression equation for predicting the stable weekly VKA dose was: square root of the VKA daily dose (mg) = (6.389 - [0.007 × age in years]-0.283 × [1, if gender male; else 0] + 0.647 × [1, if VKA = acenocoumarol; 0, if VKA = warfarin] + 0.447 × [1, if on ACE inhibitor; else 0] -0.767 × [1, if *VKORC1* -1639GA; else 0] -1.443 × [1, if *VKORC1* -1639AA; else 0] +0.326 × [1, if *CYP4F2*3* CT; else 0] +0.333 × [1, if *CYP4F2*3* TT; else 0] -0.852 × [1, if *CYP2C9*3* AC; else 0] -3.122 × [1, if *CYP2C9*3* CC; else 0]).

Marginal effects

The marginal effects of the mutant genotypes showed that all the studied polymorphisms except for *CYP2C9*2* rs1799853 were significant determinants of weekly VKA dose requirement at the Bonferroni-corrected threshold

Table 2. Genotype and allele frequencies for polymorphisms in pharmacogenes in 506 South Indian patients.

Gene, SNP	Genotypes	Frequency (%)	Allele	Frequency (%)	†HWE	Mean daily dosage, mg (±SD)	‡p-value	OR (95% CI)	§p-value
VKORC1, rs9923231	GG	408 (80.63)	G	905 (89.5)	0.246	5.82 ± 2.24	**<0.0001**	1	
	GA	90 (17.79)	A	107 (10.5)		4.39 ± 1.53		3.44 (2.09–5.66)	**<0.001**
	AA	8 (1.58)				3.09 ± 0.59		11.13 (2.78–44.56)	**0.001**
	GA + AA					4.29 ± 1.52	**<0.0001**	3.78 (2.34–6.14)	**<0.001**
*CYP4F2*3*, rs2108622	CC	153 (30.24)	C	556 (54.9)	0.962	5.09 ± 1.88	**0.0108**	1	
	CT	250 (49.41)	T	456 (45.1)		5.70 ± 2.23		0.60 (0.39–0.91)	**0.016**
	TT	103 (20.36)				5.8 ± 2.49		0.67 (0.40–1.13)	0.135
	CT + TT					5.73 ± 2.31	**0.0028**	0.62 (0.42–0.92)	**0.017**
*CYP2C9*2*, rs1799853	CC	471 (93.08)	C	977 (96.5)	0.42	5.54 ± 2.23	0.998	1	
	CT	35 (6.92)	T	35 (3.5)		5.53 ± 1.83		1.09 (0.57–2.14)	0.802
	TT	0 (0.0)				–			
	CT + TT					5.53 ± 1.83	0.998		
*CYP2C9*3*, rs1057910	AA	423 (83.60)	A	926 (91.5)	0.708	5.80 ± 2.22	**<0.0001**	1	
	AC	80 (15.81)	C	86 (8.5)		4.31 ± 1.51		3.59 (2.16–5.95)	**<0.001**
	CC	3 (0.59)				1.63 ± 0.23		5.30E+06	0.971
	AA + CC					4.21 ± 1.57	**<0.0001**	3.96 (2.40–6.53)	**<0.001**

Significant p-value < 0.05 is indicated in bold.
†p-value for: exact test of Hardy–Weinberg equilibrium.
‡One-way ANOVA test.
§Ordered logit regression analysis.
CI: Confidence Intervals; HWE: Hardy–Weinberg equilibrium; OR: Odds ratio; SD: Standard deviation.

VKORC1	*CYP4F2*	*CYP2C9*					
-1639G>A	**3*	**1/*1*	**1/*2*	**1/*3*	**2/*2*	**2/*3*	**3/*3*
GG	**CC**	STD 38.3	STD 38.9	LD 25.6	–	–	VLD 10.5
GG	**CT**	HD 44.3	HD 48.4	STD 31.9	–	STD 37.3	VLD 11.7
GG	**TT**	HD 46.6	STD 35.5	STD 30.5	–	STD 33.4	–
GA	**CC**	STD 32.5	STD 34.5	LD 27.1	–	–	–
GA	**CT**	STD 31.9	STD 32.8	STD 28.3	–	HD 36.9	–
GA	**TT**	STD 30.6	–	LD 26.4	–	–	–
AA	**CC**	LD 19.8	STD 21	–	–	–	–
AA	**CT**	LD 22.8	LD 25.9	–	–	–	–
AA	**TT**	–	–	–	–	–	–

Figure 1. Crosstabulation of mean PT/INR-based weekly vitamin K antagonist dose (in milligrams) for all genotype combinations.
HD: High dose, LD: Low dose; STD: Standard dose; VLD: Very low dose.

Table 3. Non-genetic and genetic models for predicting vitamin K antagonist dosing.

Variable	Non-genetic model			Genetic model		
	β coefficient	Standard error	p-value	β coefficient	Standard error	p-value
*VKORC1*_GA	–	–	–	-0.767	0.12	**<0.0011**
*VKORC1*_AA	–	–	–	-1.443	0.38	**0.0002**
*CYP2F4*3*_CT	–	–	–	0.326	0.11	**0.0030**
*CYP2F4*3*_TT	–	–	–	0.333	0.14	**0.0143**
*CYP2C9*3*_AC	–	–	–	-0.852	0.13	**<0.0011**
*CYP2C9*3*_CC	–	–	–	-3.122	0.61	**<0.0011**
Age	-0.01	0.00	**0.0073**	-0.007	0.00	0.0595
Male gender	-0.31	0.11	**0.0041**	-0.283	0.10	**0.0033**
Warfarin/acenocoumarol	0.66	0.11	**<0.0011**	0.647	0.10	**<0.0011**
ACE inhibitors	0.44	0.18	**0.014**	0.447	0.16	**0.0061**
Constant	6.52	0.22	**<0.0011**	6.389	0.22	**<0.0011**
R^2	0.105		**<0.0011**	0.2890		**<0.0011**
Adjusted R^2	0.098			0.2747		

Significant p-value < 0.05 is indicated in bold.
ACE: Angiotensin-converting enzyme.

Table 4. Marginal effects of polymorphisms on average vitamin K antagonist weekly dose requirement.

Genotype	Overall dosing categories, n = 506	p-value	Low-dose category, n = 62	p-value	Intermediate-dose category, n = 325	p-value	High-dose category, n = 119	p-value
*VKORC1*_GA	-0.51 (-0.94 to -0.21)	**<0.0001**	-0.01 (-0.14 to 0.03)	0.444	-0.02 (-0.09 to 0.00)	0.088	-0.12 (-0.9 to 0.06)	0.259
*VKORC1*_AA	-2.84 (-6.11 to -0.81)	**<0.0001**	0.05 (-0.14 to 0.68)	0.457	-1.11 (-2.64 to -0.23)	**<0.0001**	–	–
*VKORC1*_GA + AA	-0.6 (-1.04 to -0.27)	**<0.0001**	-2.98E-05 (-0.08 to 0.07)	0.969	-0.04 (-0.12 to -1.14E-03)	0.015	-0.15 (-1.03 to 0.05)	0.218
*CYP2F4*3*_CT	0.12 (0.01 to 0.32)	**0.003**	0.09 (0 to 0.34)	0.044	4.43E-4 (-0.02 to 0.03)	0.776	1.52E-3 (-0.07 to 0.12)	0.803
*CYP2F4*3*_TT	0.1 (0 to 0.35)	0.029	0.02 (-0.05 to 0.28)	0.434	-1.87E-4 (-0.04 to 0.03)	0.880	0.23 (0.01 to 0.73)	**0.012**
*CYP2F4*3*_CT + TT	0.13 (0.02 to 0.33)	**<0.0001**	0.06 (-1.07E-03 to 0.27)	0.090	6.65E-04 (-0.01 to 0.03)	0.712	0.02 (-0.03 to 0.21)	0.346
*CYP2C9*3*_CT	0.02 (-0.06 to 0.27)	0.489	-2.47E-3 (-1.4 to 1.08)	0.932	0.01 (-0.01 to 0.11)	0.316	0.08 (-0.09 to 0.73)	0.338
*CYP2C9*2*_CT + TT	0.01 (-0.09 to 0.21)	0.694	0.04 (-0.75 to 1.60)	0.713	2.79E-03 (-0.03 to 0.07)	0.639	0.03 (-0.17 to 0.59)	0.549
*CYP2C9*3*_AC	-0.81 (-1.36 to -0.4)	**<0.0001**	-1.36E-4 (-0.09 to 0.08)	0.938	-0.14 (-0.29 to -0.04)	**<0.0001**	0 (-0.64 to 0.50)	0.904
*CYP2C9*3*_CC	-9.06 (-18.18 to -3.09)	**<0.0001**	-0.71 (-2.07 to -0.06)	**0.008**	–	–	–	–
*CYP2C9*3*_AC + CC	-0.92 (-1.49 to -0.48)	**<0.0001**	-0.03 (-0.21 to 0.01)	0.251	-0.13 (-0.27 to -0.04)	**<0.0001**	-0.01 (-0.8 to 0.43)	0.763

Boneferroni-corrected p-value < 0.0125 indicated in bold.
'-' signifies a lack of samples in that particular category.

p-value < 0.0125 (Table 4). The CC genotype carriers of *CYP2C9*3* showed a significantly reduced average weekly dose requirement of -9.06 mg ($p < 0.0001$) in the overall category and -0.71 mg in the low-dose category ($p = 0.008$). The AC genotype of *CYP2C9*3* and the AA genotype of *VKORC1* rs9923231 contributed to reduced average weekly dose requirements of -1.11 mg and -0.14 mg, respectively, in the intermediate-dose category ($p < 0.0001$). In the high-dose category, the *CYP2F4* TT genotype contributed to a significantly increased weekly dose requirement of 0.23 mg ($p = 0.012$).

Vitamin K antagonist-induced adverse effects

The association of genotypes among patients with the risk of VKA-induced bleeding events is shown in Table 5. Bleeding events were recorded in 24 patients (4.74%). Of these, 7 patients (29%) carried the *VKORC1* rs9923231 GA or AA genotype associated with a low dose whereas the remaining 17 (71%) had the GG genotype associated

Table 5. Association of genotypes with risk of vitamin K antagonist-induced bleeding events.

Gene, SNP	Genotypes	Present (%)	Absent (%)	Total (%)	†p-value	‡OR (95% CI)	‡p-value
VKORC1, rs9923231	GG	17 (70.8)	391 (81.1)	408 (80.6)	0.021	Ref	
	GA	5 (20.8)	85 (17.6)	90 (17.8)		1.33 (0.47–3.79)	0.586
	AA	2 (8.3)	6 (1.2)	8 (1.6)		8.01 (1.39–46.01)	**0.019**
*CYP4F2*3*, rs2108622	CC	8 (33.3)	145 (30.1)	153 (30.3)	0.321	Ref	
	CT	14 (58.3)	236 (49.0)	250 (49.4)		1.03 (0.42–2.55)	0.944
	TT	2 (8.3)	101 (21.0)	103 (20.4)		0.32 (0.07–1.58)	0.164
*CYP2C9*2*, rs1799853	CC	23 (95.8)	448 (93.0)	471 (93.1)	0.586	Ref	
	CT	1 (4.2)	34 (7.05)	35 (6.9)		0.57 (0.07–4.49)	0.594
*CYP2C9*3*, rs1057910	AA	19 (79.2)	404 (83.8)	423 (83.6)	0.064	Ref	
	AC	4 (16.7)	76 (15.8)	80 (15.8)		1.14 (0.37–3.53)	0.818
	CC	1 (4.2)	2 (0.41)	3 (0.59)		16.64 (1.08–256.20)	**0.044**

Significant p-value < 0.05 is indicated in bold
†p-value of Pearson's χ^2 test.
‡Odds ratio (95% CI) adjusted for target prothrombin time/international normalized ratio and vitamin K antagonist indication.
CI: Confidence Intervals.

with a high dose. Logistic regression analysis adjusted for the indication for the anticoagulant, target PT/INR and TTR showed a significant association of the *VKORC1* rs9923231 AA and *CYP2C9*3* rs1057910 CC genotypes with bleeding events (OR = 8.01, p = 0.019; OR = 16.64, p = 0.043, respectively). However, there were no significant gene × gene interactions between *VKORC1* rs9923231 and *CYP2C9*3* rs1057910 or with gene × enzyme inducer/inhibitor suggestive of any association with increased risk of VKA-induced bleeding events.

Discussion

In this multicentric study from Kerala, India, a large sample size of 506 patients with mechanical prosthetic heart valve implantations who achieved stable anticoagulation through treatment with vitamin K antagonists like warfarin or acenocoumarol were genotyped. Stable VKA doses were significantly different among the variant genotype carriers. Specifically, carriers with the *VKORC1* -1639G >A rs9923231 and *CYP2C9*3* rs1057910 polymorphisms required a lower dose whereas *CYP4F2*3* carriers required a higher dose. Multiple regression and marginal effects analyses indicated that genetic variants were important determinants for VKA dosing with a larger effect size compared with non-genetic factors (9.8% vs 27.47%).

Researchers assessing South Indian patients with various cardiological and neurological phenotypes have investigated the associations between polymorphisms of *VKORC1*, *CYP2C9* and *CYP4F2* as well as other genes such as *GGCX* with warfarin dose requirements [14–21]. Similar genetic associations with acenocoumarol dose requirements have also been reported [22–26]. Notably, genetic variants in the *VKORC1* and *CYP2C9* genes have been consistently associated with low dose requirements in Indian populations and the current data also demonstrated a similar trend. However, the association of *CYP4F2*3* polymorphism with a high dose requirement has been contentious due to contradictory reports from the Indian population [15,16,18,24,25]. The present data showed that the *CYP4F2*3* allele was associated with a statistically significant increase in weekly dose in patients in the high-dose category and this is the largest cohort to date to have this association reported in any Indian population. In this study sample, the MAF of the *CYP4F2*3* allele was as high as 45.1%, which is higher than the global population, where the MAF ranges 10–40%. And despite having a high MAF, due to the small effect size of this allele, a large sample size was required to detect the association between the *CYP4F2*3* allele and anticoagulant dose requirement.

Unlike the other tier 1 pharmacogenetic variants in *VKORC1* and *CYP2C9* genes recommended by the Association for Molecular Pathology (AMP) Pharmacogenetics Working Group, the functional impact of the *CYP4F2*3* allele is not straightforward, as it does not affect mRNA transcription or protein enzymatic activity; however, it reportedly decreases protein levels via a decreased rate of protein translation or an increased rate of protein degradation [27,28]. At the protein level, CYP4F2 counteracts the effects of VKORC1 by metabolizing and limiting vitamin K availability and the presence of the *CYP4F2*3* allele is postulated to reduce the capacity to metabolize vitamin K.

The lack of interaction between the *VKORC1* rs9923231 and *CYP4F2*3* alleles indicate that the functional effects of the alleles on dose are mutually exclusive and supports the inclusion of the *CYP4F2*3* allele into pharmacogenetic test panels and dosing algorithms.

The functionality of an allele exerts an effect on drug dosage determination, and this has been demonstrated empirically for the *CYP2C9*2* and **3* alleles using *in vivo* and *in vitro* studies. According to the PharmGKB Clinical Annotation, the *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* alleles are labeled "normal function", "decreased function" and "no function" alleles, respectively, by the CPIC. The reason for the *CYP2C9*2* allele not having a significant effect on dosing in the current study could be the low MAF observed in the South Asian population. In this sample of 506 patients, the observed MAF of the *CYP2C9*2* decreased-function allele was 3.5%. This is in contrast to reports from Caucasian populations that have reported allele frequencies that range 10.7–12.5% [29]. On the contrary, the *CYP2C9*3* no-function allele, which showed a significant association with a low dose, was found to have a MAF of 8.5% in the present study, which is similar to that reported in Caucasian populations, which range 7.4–8.5% [29]. Taken together, this indicates that the *CYP2C9*3* allele is a relatively more important pharmacogenetic variant for the South Indian population than the *CYP2C9*2* allele, since it has a statistically significant functional effect on dose and has a higher allele frequency.

The original International Warfarin Pharmacogenetics Consortium (IWPC) warfarin-dosing algorithm was developed based on data that arose largely from people of European ancestry, East Asia and African–Americans. The IWPC algorithm that accounted for 47% of warfarin dose variability included clinical variables such as age, amiodarone use, weight, height, use of *CYP2C9* inducers and race/ethnicity, along with genetic factors such as *VKORC1* -1639G >A rs9923231, *CYP2C9*2* rs1799853 and *CYP2C9*3* rs1057910, with *CYP4F2*3* rs2108622 included as an optional factor in the most recent update in 2017 [6]. However, when genetic factors were tested for association with VKA dose in the present sample, the genetic model could only predict 27.47% of the variability in PT/INR-based VKA dosing in patients from Kerala, India. Interestingly, the predictive ability of the reported genetic models assessed by the R^2 statistic showed a wide discrepancy among different Indian studies, ranging 36.1–67% in multiple regression models fitted with different sets of genetic and non-genetic variables. The authors opine that the variability may be influenced by various factors, such as differences in the MAF in diverse subpopulations of India, interaction with concomitant drugs with different anticoagulant indications or even due to selection bias that could have influenced the proportion of anticoagulant-sensitive or -resistant patients included the study sample. In a previous study, the authors used the 1000 Genomes dataset to compare the genotype frequencies of warfarin pharmacogenes in the South Asian population to other global populations [30]. Significant interpopulation differences in MAFs of variants in warfarin pharmacogenes were found and could underlie the differences in the proportion of variability explained by these genetic variants in other populations. Moreover, the number of independent variables included in the model can also influence the effect size, where the inclusion of multiple genetic variants that are in linkage disequilibrium could have substantially inflated the R^2 values of these models. For instance, Limdi *et al.* has shown that *VKORC1* SNPs that were in nearly complete linkage disequilibrium were essentially equally predictive of the warfarin dose explained by R^2 across Asian, Black and White racial groups [5].

A unique feature of this study is that the sample consisted of patients who used both warfarin and acenocoumarol, which was equated using a transition factor and combined into a single-dependent variable. This enabled us to simulate a clinical scenario where either warfarin or acenocoumarol was prescribed to patients with mechanical heart valve implants in our clinics. Further, the genetic model fitted in the present study included only the four CPIC-recommended genetic variants that enabled comparison to the IWPC genetic model. It is likely that an expanded model with the inclusion of variants identified through population-specific genome-wide association studies could help explain the entire proportion of variability contributed by genetic variants in this population. For instance, in a previous study, we identified *VKORC1* rs7294 polymorphism as a potential mirSNP that could contribute to interindividual as well as interpopulation variability observed in warfarin dosing for the South Asian population on the basis of interpopulation linkage disequilibrium variation, *in silico* analysis and evidence from genome-wide association studies and candidate gene association studies [30]. The use of concomitant drugs that act as *CYP2C9* enzyme inducers or enhancers influences VKA dosing, and ACE inhibitors were a significant factor that affected VKA dosing among patients with mechanical heart valve implants. A study on first-time users of oral anticoagulants reported a significantly increased likelihood of an elevated INR (>3) with concomitant use of antihypertensives like ACE inhibitors among Indian patients [31].

Despite the low frequency of *VKORC1* rs9923231 AA and *CYP2C9*3* rs1057910 CC genotypes among South Indian patients (1.58% and 0.59%, respectively), these genotypes seemed to have a large impact on VKA-induced

bleeding events (ORs: 8.29 and 17.04, respectively). Due to the rarity of these variants, we could not previously identify this effect in a smaller cohort of 222 samples where we analyzed the influence of *VKORC1* rs9923231 alone on warfarin dosing [21]. Since these polymorphisms are rare in the South Indian population, the large CIs indicate the need for larger sample sizes to replicate the associations with bleeding events.

Conclusion

These data show that the addition of pharmacogenetic variants to the dosing model could improve the predictability of dose categorization among South Indian patients with mechanical prosthetic heart valves. The implications of implementing such genotype-guided initial VKA dosing may be to reach the target maintenance dose faster and with fewer adverse effects, especially since a majority of the South Indian patient population is genetically predisposed to require a high dose of VKAs. This data also adds to the existing literature on pharmacogenomic studies of vitamin K antagonists like warfarin and acenocoumarol, which will be useful to gather a bird's-eye view of the dosing variability seen among diverse global populations.

Future perspective

Oral VKAs prescribed globally have been the mainstay of anticoagulation therapy for the prevention of thromboembolic events for many decades and may continue to be used for a long time to come. Testing the pharmacogenetic variants and quantifying their relative effects on dosing is essential both at the patient level and at the population level to understand the variability in anticoagulant dose requirement that is frequently observed by clinicians. Hence, it is important to formulate pharmacogenetic studies that will help quantify the inherent dosing variability seen in diverse populations to suit personalized anticoagulant therapy for patients. This knowledge can be used to develop population-specific or even phenotype-specific pharmacogenetic algorithms with improved predictive ability for VKA dosing and to reduce adverse effects in patients from diverse ethnic populations.

Summary points

- Following the genotyping of Clinical Pharmacogenetics Implementation Consortium-recommended pharmacogenetic variants, significant differences in the mean prothrombin time/international normalized ratio-based vitamin K antagonist (VKA) daily doses were observed for the *VKORC1* -1639G >A rs9923231, *CYP2C9*3* rs1057910 and *CYP4F2*3* rs2108622 genotypes.
- The addition of the pharmacogenetic data into the VKA-dose prediction model improved the value of the coefficient of determination (adjusted R^2) from 9.8% to 27.47%.
- The pharmacogenetic variants showed a significant discriminatory ability to assign the patients to low-, intermediate- or high-dose categories.
- The low-dose requiring genotypes of *VKORC1* -1639G >A rs9923231 and *CYP2C9*3* rs1057910 variants were also associated with the risk of VKA-induced bleeding events.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pgs-2022-0014

Author contributions

L Koshy: conception of study design, genotyping, data interpretation and analysis; manuscript writing, revision and correction. Raghu VB: DNA isolation, genotyping, data interpretation and analysis. Madhuma M: DNA isolation, genotyping, data interpretation and analysis. Midhuna PB: DNA isolation, genotyping, data interpretation and analysis. P Kishor: DNA isolation, genotyping, data interpretation and analysis. PR Sudhakaran: data interpretation and analysis, manuscript revision and correction. J Abdullakutty: patient sample and clinical data acquisition, manuscript revision and correction. K Venugopal: patient sample and clinical data acquisition, manuscript revision and correction. G Zachariah: patient sample and clinical data acquisition, manuscript revision and correction. PP Mohanan: patient sample and clinical data acquisition, manuscript revision and correction. S Harikrishnan: patient sample and clinical data acquisition, conception of the work, revision and final approval of the manuscript and funding acquisition. Sanjay G: patient sample and clinical data acquisition, conception of the work, writing, revision and final approval of the manuscript and funding acquisition.

Acknowledgments

We thank all the patients who participated in this study. We thank the National Heart Failure Biobank supported by the Indian Council of Medical Research (ICMR), New Delhi, India, File no. 55/4/5/CARE-HF/2018-NCD-II, for providing the clinical data and samples. We thank Dr. GM Nair, Dr. A Jayakumaran Nair and Dr. A Gangaprasad of Inter-University Centre for Genomics and Gene Technology, University of Kerala, for providing the laboratory facilities. We acknowledge Mr. Vineeth CP, Mr. Kochumoni R and the coordinators at the collaborating hospitals who facilitated the sample and data collection.

Financial & competing interests disclosure

This study was funded by the Cardiological Society of India, Kerala Chapter, ref. no. CSIK/KER/SREC/2016/03. The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Written informed consent from all participants was obtained after the study protocol was explained. The study protocol conformed to the guidelines set by the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences & Technology (SCTIMST), Trivandrum, India, ref. no. SCT/IEC/1013/December-2016 (4 May 2017), and the Independent Ethics Committee of the Cardiological Society of India-Kerala Chapter.

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