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PHARMACOGENETICS

VKORC1 −1639G>A and *CYP2C9*3* are the major genetic predictors of phenprocoumon dose requirement

Helene Puehringer · Ralph M. Loreth · Gudrun Klose · Brigitte Schreyer · Walter Krugluger · Barbara Schneider · Christian Oberkanins

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Abstract
Purpose Phenprocoumon, similar to other coumarin-derived anticoagulants, is associated with a large variation in the individual dose requirement to achieve stable anticoagulation. Polymorphisms in the vitamin K epoxide reductase complex subunit 1 (VKORC1) and the liver enzyme cytochrome P450 2C9 (CYP2C9) effectively account for the variability in warfarin and acenocoumarol response but are less well-defined pharmacogenetic predictors in phenprocoumon therapy.
Methods A retrospective study was performed on 185 outpatients attending anticoagulation clinics in Austria and Germany. These patients were genotyped for the *VKORC1* −1639G>A and 3730G>A polymorphisms as well as for the *CYP2C9 *2* and **3* polymorphisms using a reverse hybridisation-based teststrip assay.
Results The *VKORC1* −1639A allele, which was present at a frequency of 41.4% in the study cohort, significantly reduced the mean weekly phenprocoumon dose by 3 mg (19%) in the heterozygous and by 6.7 mg (43%) in the homozygous state compared to wild-type carriers (15.5± 6.8 mg, $p<0.0001$). A stepwise multiple regression analysis revealed that *VKORC1* −1639G>A, age and *CYP2C9*3* were the major independent determinants of phenprocoumon dose, accounting for 14.2, 9.1 and 4.7% of its variability, respectively ($p\leq0.0007$). The *CYP2C9*2* polymorphism had a marginal influence (1.4%) and failed to reach statistical significance ($p=0.062$). The *VKORC1* 3730G>A genotype had no additional predictive power for individual dose variability.
Conclusion Similar to warfarin and acenocoumarol, the *VKORC1* −1639G>A polymorphism had the highest impact on the maintenance dose of phenprocoumon. The factor age was the second most important predictor and explained a greater percentage of the variability than *CYP2C9* genotype.

Keywords Coumarins · *CYP2C9* · Dose variability · Phenprocoumon · *VKORC1*

H. Puehringer (✉) · C. Oberkanins
ViennaLab Diagnostics GmbH,
Gaudenzdorfer Guertel 43-45,
1120 Vienna, Austria
e-mail: puehringer@viennalab.co.at

R. M. Loreth · G. Klose · B. Schreyer
Clinical Haemostaseology, Westpfalz-Klinikum GmbH,
Kaiserslautern, Germany

W. Krugluger
Department of Clinical Chemistry, Rudolfstiftung Hospital,
Vienna, Austria

W. Krugluger
Department of Clinical Chemistry, Donauspital,
Vienna, Austria

B. Schneider
Section for Medical Statistics and Informatics,
Medical University Vienna,
Vienna, Austria

Introduction

The vitamin K antagonists (VKAs) warfarin, acenocoumarol and phenprocoumon are widely prescribed and highly effective oral anticoagulants for the long-term treatment and prophylaxis of a number of thromboembolic disorders. The management of anticoagulation with coumarins is complicated by their narrow therapeutic window, the high inter-individual dose variability and the increased risk of hemorrhagic side effects in patients requiring a low dose. To assure an efficient and safe therapy, patients

require an individual dose adjustment and constant monitoring of the international normalised ratio (INR). Warfarin is the oral anticoagulant of choice in most countries, particularly in North America, UK and Scandinavia, while acenocoumarol and phenprocoumon are frequently prescribed in many continental European countries.

Since the discovery of the target enzyme VKORC1 [1, 2], several rare amino acid substitutions leading to warfarin resistance have been described to date [2–5]. Furthermore, common single nucleotide polymorphisms (SNPs) in non-coding regions of the *VKORC1* gene are known to be associated with altered drug sensitivity [6–8]. Among these, the tightly linked promoter variant −1639G>A and the 1173C>T substitution in intron 1 are the most useful tag-SNPs for the identification of the low-dose haplotype group A (variant) and the high-dose haplotype group B (wild-type) described by Rieder et al. [7].

Whereas *VKORC1* polymorphisms have a relatively similar pharmacodynamic effect on all VKAs, the contribution of cytochrome P450 2C9 (CYP2C9) to the clearance of each anticoagulant appears to be substantially different. CYP2C9 is mainly responsible for the metabolic clearance of the *S*-enantiomere, which shows the highest anticoagulation activity in the racemic mixture of coumarins. About 40% of ingested phenprocoumon are excreted unchanged, whereas warfarin and acenocoumarol are almost completely metabolised mainly by CYP2C9-driven hydroxylation. The biologically more active S-phenprocoumon is cleared to 65% only by CYP2C9 and to 35% by the less polymorphic CYP3A4 [9]. The defective CYP2C9*2 and *3 variants are causally linked to a reduced dose requirement for warfarin [10–13], and a similar effect of CYP2C9*3 on acenocoumarol has been repeatedly reported [14–16]. The metabolism of phenprocoumon seems to be less influenced by *CYP2C9* genotypes [17, 18]. The combined effect of the *VKORC1* and *CYP2C9* alleles on dose variability, severe overanticoagulation and time to achieve stable anticoagulation has been extensively studied in patients treated with warfarin [19–22] and to a lesser extent in acenocoumarol-treated patients [15, 23, 24]. However, there is still only limited and somewhat controversial information available on the relative contributions of *VKORC1* and *CYP2C9* polymorphisms to the clinical outcome of phenprocoumon-treated patients [25–28]. Moreover, in contrast to warfarin, for which several dosing algorithms have been developed in recent years [29–33], no pharmacogenetic-based predictive rules for determining the initial dose of phenprocoumon treatment are currently established.

Here, we report the results of our investigation on the extent to which genetic polymorphisms in the *VKORC1* promoter (−1639G>A) and the 3′-untranslated region (3730G>A)—the latter defining a subtype of the high-dose haplotype B [7, 8]—as well as CYP2C9*2 and *3 contribute to individual dose variability in phenprocoumon therapy.

Methods

Patients

For this retrospective study, we recruited 185 patients on long-term maintenance therapy with phenprocoumon (Marcumar) between April 2007 and December 2008. All outpatients ≥18 years with a stable anticoagulation status who were being monitored in clinics in Austria (Rudolfstiftung Hospital, Vienna; $n=76$) and Germany (Westpfalz-Klinikum, Kaiserslautern; $n=109$) were eligible for the study. No exclusion criteria were applied. At the time of admission most study participants were being co-treated with ß-blockers, angiotensin-converting enzyme inhibitors, digitalis glycosids, diuretics, simvastatin or antidiabetic drugs due to various age-related co-morbidities (mean age of patients 69.6 years). Mean weekly phenprocoumon doses were calculated after stable anticoagulation was achieved, which was defined as INR measurements within the target INR of 2.5±0.5 at constant phenprocoumon dosing for at least 3 consecutive clinic visits. For the vast majority of patients, INR values were recorded every 3 weeks. Blood samples were taken during routine controls, and data on age, gender, weight and required phenprocoumon dose were collected for each patient. All subjects provided informed consent to participate in the study.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the GEN^{X}TRACT Blood DNA Extraction System (ViennaLab Diagnostics, Vienna, Austria). Genotyping for the *VKORC1* −1639G>A and 3730G>A SNPs as well as for the *CYP2C9* allelic variants **2* (430C>T) and **3* (1075A>C) was performed by PCR followed by reverse-hybridisation as described for teststrip assays [34]. Briefly, DNA fragments spanning the four SNPs were amplified and biotinylated in a multiplex PCR using primers described elsewhere [6, 13, 35]. Amplicons were hybridised for 30 min at 45±0.5°C to a membrane teststrip presenting parallel aligned allele-specific oligonucleotides for each of the two *VKORC1* [−1639G>A: TTGGCC(G/A)GGTGCG; 3730G>A: ATACCC(G/A)CACATG] and *CYP2C9* [**2*: GAGGAC(C/T)GTGTTC; **3*: AGATAC(A/C)TTGACC] polymorphisms. After a series of stringent washes, specifically bound PCR fragments were detected using a streptavidin–alkaline phosphatase conjugate and colour substrates (BCIP/NBT reaction). The entire hybridisation

and detection procedure was carried out either manually or fully automated using a temperature-controlled teststrip processor (profiBlot II T 48; TECAN, Groedig, Austria). Reference DNA samples previously typed by direct DNA sequencing were available for all SNPs and were used for performance control of the assay.

Statistical analysis

For a description of age, weight and dose, we computed the mean, standard deviation (SD), median and minimum and maximum values. Pearson correlation coefficients were calculated to estimate the associations between age, weight and dose. The quality of the genotype data was assessed by testing for Hardy–Weinberg equilibrium. Genotypes were coded according to the number of variant alleles per patient (0, 1, 2). Associations between different genotypes or between genotypes and sex were analysed by contingency tables in combination with a χ^2 test. The dependency of age, weight and dose on different genotypes or sex was analysed using one-way analysis of variance (ANOVA) with Tukey's post hoc test, if appropriate. We used a stepwise linear regression model to estimate the impact of genotypes, age, weight and sex on the dose. Due to the skewed distribution, the square root-transformed dose values were used as the dependent variable in the model. For all tests a p value <0.05 was considered to be statistically significant. Because of the retrospective nature of the data, all statistical test results have to be interpreted with caution. SAS ver. 9.2 statistical software (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Population characteristics

The study population consisted of 185 European Caucasians with stable control of phenprocoumon therapy. Age, weight and sex distribution, indications for anticoagulation and *VKORC1* and *CYP2C9* genotype frequencies of the study participants are summarised in Table 1. Among these patients, the mean phenprocoumon dose required to maintain a therapeutic INR of 2–3 was 12.9 mg/week, but individual doses ranged from 1.5 to 34.5 mg/week, demonstrating a 23-fold inter-patient variability. Testing for *VKORC1* SNPs among the study participants revealed a frequency of 41.4% for the low-dose haplotype A-determining −1639A allele and 37.3% for the minor 3730A allele. The allele frequencies for *CYP2C9*2* and **3* were 11.9 and 8.1%, respectively. The observed genotype frequencies for the *VKORC1* and *CYP2C9* SNPs showed no deviation from Hardy–Weinberg equilibrium (data not shown). Pairwise comparison of genotype frequencies revealed a significant linkage between the −1639G>A and 3730G>A loci in the *VKORC1* gene (χ^2 test $p<0.0001$). All patients homozygous for −1639AA ($n=33$) were found to be wild-type (GG) at the 3730 locus, whereas all homozygous 3730AA carriers ($n=29$) were wild-type (GG) at the −1639 locus. All other genotypes did not show any statistically significant distribution. Demographic factors, such as age, weight and gender, did not vary significantly among different *VKORC1* and *CYP2C9* genotypes except for *VKORC1* 3730AA, which was significantly over-represented in younger patients ($p<0.02$).

Impact of demographic variables and *VKORC1* and *CYP2C9* polymorphisms on phenprocoumon dose

Correlation analysis of the weekly phenprocoumon dose revealed a statistical significant negative correlation with age ($r=-0.35$) and a positive correlation with body weight ($r=0.22$). No significant differences were present between males and females.

The influence of genetic polymorphisms on the mean weekly dose needed to achieve stable anticoagulation was initially investigated by univariate analysis in patients not adjusted for age and weight (Fig. 1). Homozygous carriers of the *VKORC1* −1639A allele required a significantly lower dose (8.8±5.3 mg) than heterozygous GA (12.5±5.3 mg) or wild-type GG (15.5±6.8 mg) carriers (Fig. 1a). An inverse correlation was observed for the *VKORC1* 3730G>A polymorphism, with the average dose for homozygous AA carriers being higher (15.9±7.5 mg) than that for heterozygous GA (13.2±6.2 mg) or homozygous GG (11.5±5.5 mg) carriers (Fig. 1b). However, the difference was only significant between homozygous GG and AA genotypes and could be explained by (1) the high linkage of both *VKORC1* loci and (2) the significantly higher proportion of AA genotypes in younger patients needing a higher dosage.

Irrespective of the *VKORC1* genotype the presence of at least one *CYP2C9*3* allele in patients substantially decreased the weekly administered mean phenprocoumon dose to 10.1±4.8 mg compared to 13.9±6.2 mg in *CYP2C9*1*1* wild-types. In carriers with one *CYP2C9*2* allele, the dose reduction, to 12.3±6.8 mg, was much less pronounced. Only one homozygous patient for each *CYP2C9* variant and two compound heterozygous patients were found in our cohort (Fig. 1c; Table 2). However, none of the dose differences between *CYP2C9* genotypes reached statistical significance due to the relatively small sample size.

The impact of the combined *VKORC1* −1639G>A and *CYP2C9* genotypes on the mean weekly phenprocoumon dose requirement is shown in Table 2. In all *VKORC1*

Table 1 Baseline characteristics of the study population

Characteristics	Number of genotyped patients, n = 185 (%)	mean (±SD), range
Sex		
Male	94 (51)	
Female	91 (49)	
Age (year)		
Male		68.6 (±13.0), 19–93
Female		70.6 (±14.0), 21–90
Weight (kg)		
Male		87.1 (±16.4), 64–155
Female		74.9 (±18.2), 43–166
Phenprocoumon dose (mg/week)		
Male		13.6 (±5.8), 3–34.5
Female		12.2 (±6.7), 1.5–31.5
Reason for anticoagulation		
Atrial fibrillation	115 (62.2)	
Thromboembolism	53 (28.6)	
Prosthetic heart valve	9 (4.9)	
Cardiomyopathy	4 (2.2)	
Others	4 (2.2)	
Genotype frequencies		
VKORC1		
−1639 GG	65 (35.2)	
−1639 GA	87 (47.0)	
−1639 AA	33 (17.8)	
3730 GG	76 (41.1)	
3730 GA	80 (43.2)	
3730 AA	29 (15.7)	
CYP2C9		
*1*1*	115 (62.2)	
*1*2*	40 (21.6)	
*1*3*	26 (14.1)	
*2*2*	1 (0.5)	
*2*3*	2 (1.1)	
*3*3*	1 (0.5)	

SD, Standard deviation

genotype groups, carriers of the *CYP2C9*3* allele required a considerable lower dose than **2* carriers compared to carriers of the *CYP2C9* wild-type. The dose-reducing effect conferred by the **3* variant was more evident in −1639GG patients (−4.8 mg) than in those with the GA (−3.2 mg) or AA (−1.8 mg) genotypes.

Multiple linear regression modelling

A multiple linear regression analysis was performed to test for individual contributions of genotypes and demographic factors to the variance in maintenance dosage. Variables found to be associated with phenprocoumon dose by univariate analysis were entered into the regression model. The stepwise analysis revealed a statistically significant contribution of *VKORC1* −1639G>A, age and *CYP2C9*3* to the variability of the square root-transformed phenprocoumon dose (Table 3). The model demonstrated that among all investigated parameters *VKORC1* −1639G>A most strongly determined the weekly dose (r^2=0.142), followed by patient age (r^2=0.091) and *CYP2C9*3* (r^2= 0.047), jointly explaining 28% of the observed variability. *CYP2C9*2* and weight turned out to have a marginal and insignificant influence on individual dose requirement, adding only 1.4 and 1.2%, respectively. *VKORC1* 3730G>A and gender were excluded, as they did not meet the significance criteria for entry into the model. Altogether, the full model comprising five covariates explained 31% of the variability observed in the square root-transformed phenprocoumon doses (r^2=0.31; Table 3).

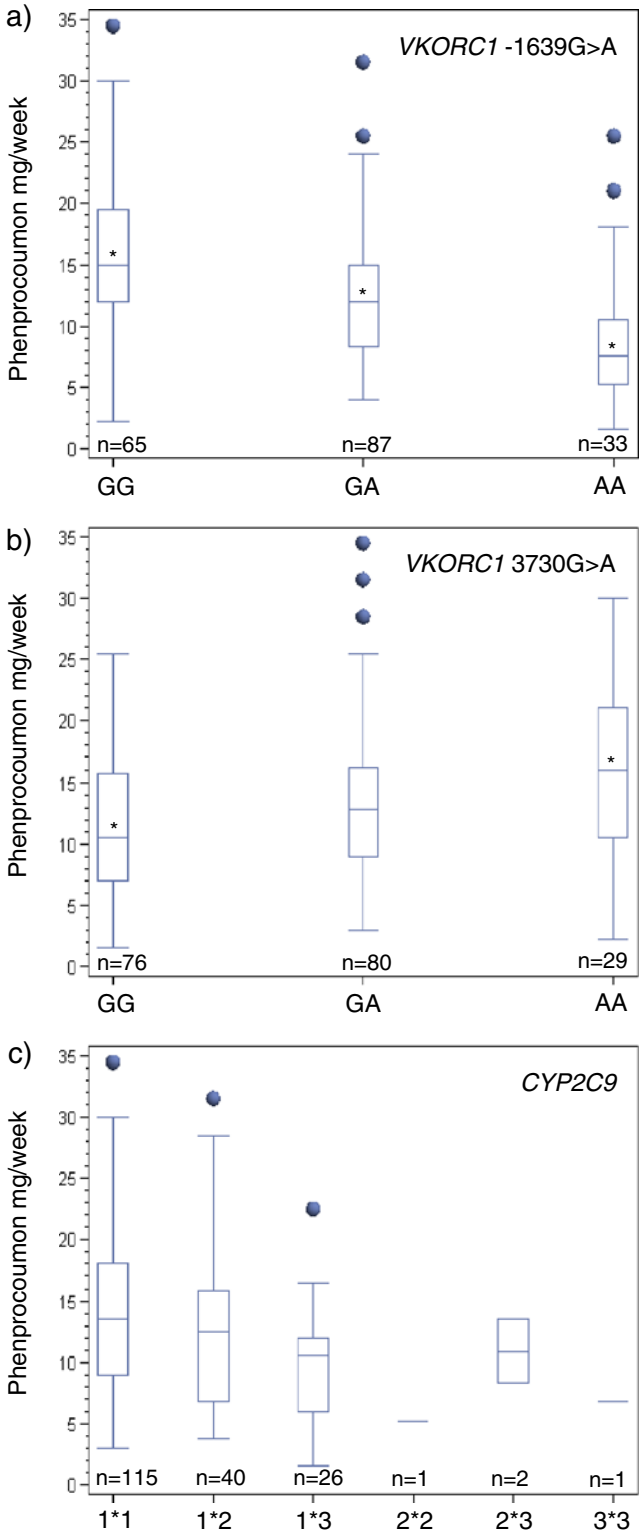

Fig. 1 *Box and whisker plots* showing the distribution of weekly phenprocoumon doses based on different *VKORC1* −1639G>A (**a**), *VKORC1* 3730G>A (**b**) and *CYP2C9* (**c**) genotypes. *Boxes* Median and upper and lower quartiles, respectively. The maximum length of each whisker is 1.5× the interquartile distance. *Points falling outside* the plots show up as outliers. *Asterisks above the median* indicate statistically significant differences

Discussion

Among our ethnically homogenous patient group we observed an up to 23-fold variation in dose requirement to achieve stable anticoagulation, which reflects the known high inter-individual variability. Our aim was to achieve an understanding of the basis for this large difference in sensitivity to phenprocoumon by evaluating the main genetic polymorphisms implicated in the drug's action and metabolism along with non-genetic parameters.

Our data corroborate the predominant role of *VKORC1* haplotypes in the anticoagulation process with all classes of coumarin derivatives. As expected, the *VKORC1* tag-SNP −1639G>A haplotype showed the greatest impact on phenprocoumon dose requirement and explained 14.2% of the dose response variability in our study. Two copies of the −1639A allele conferred the highest sensitivity to the drug, leading to a 43% lower dose requirement compared to two wild-type alleles. There is some evidence that this SNP is indeed the causal polymorphism for the haplotype A-induced sensitivity due to a reduced transcriptional activity of the promoter [36], and, consequently, a lower mRNA transcript level [7] that translates into decreased VKORC1 activity rendering the patient more susceptible to VKAs.

Genotyping for *VKORC1* 3730G>A, which defines a subtype of the high-dose haplotype B [7, 8], had no additional predictive power for individual dose variability in our study. The effect of 3730G>A on phenprocoumon dose seen in the univariate analysis may be largely caused by its linkage to −1639G>A. According to Wadelius et al. [8], the linkage disequilibrium for both SNPs is 0.39 (r^2 measurement). The fact that SNP 3730G>A failed the entry into our regression model underlines its negligible role in the phenprocoumon dose requirement. This is in contrast to findings of two other studies in which the 3730G>A SNP contributed 1.2 and 9% to the variability in warfarin dose, respectively [37, 38].

Higher age is an independent predictor of lower phenprocoumon needs. In our patient group, the factor age was the second-most important predictor and explained a greater percentage of the variability in dosage than the *CYP2C9* genotype, which is in line with previous reports on coumarin anticoagulants [26, 29]. Drug metabolism decreases with age and is probably related to a reduced ability to metabolise coumarin derivatives. Furthermore, food intake and concomitantly the intake of vitamin K decreases in elderly patients, thereby increasing the individual's coumarin sensitivity.

Somewhat conflicting results have been reported in the past in terms of the impact of *CYP2C9* genotypes on phenprocoumon dosing. While no differences were found

Table 2 Impact of the *VKORC1* −1639G>A and *CYP2C9* genotype combinations on mean phenprocoumon dose

VKORC1 −1639G>A	*CYP2C9*	Number of carriers	Mean phenprocoumon dose (mg/week)[a]
GG	**1*1*	41	16.5 (±7.4)
	**1*2*	14	15.4 (±5.6)
	**1*3*	10	11.7 (±5.3)
GA	**1*1*	58	13.3 (±4.6)
	**1*2*	17	11.7 (±7.3)
	**1*3*	9	10.1 (±3.0)
	**2*3*	2	10.9 (±3.7)
	**3*3*	1	6.8
AA	**1*1*	16	9.6 (±5.5)
	**1*2*	9	8.7 (±5.1)
	**1*3*	7	7.8 (±4.7)
	**2*2*	1	5.3

[a] Standard deviation is given in parenthesis

in two studies [27, 28], Schalekamp et al. [25] reported a significant dose difference between carriers of a *CYP2C9*2* or **3* variant and *CYP2C9*1*1* wild-type patients. In a follow-up study, this effect was shown to be modified by the *VKORC1* C1173T genotype, being most pronounced in CC carriers [26]. In our cohort, a small but significant dose-reducing effect by a single copy of the *CYP2C9*3* allele was still apparent after patients were stratified for *VKORC1* −1639G>A and age. For the *CYP2C9*2* allele, the effect was much smaller and failed to reach statistical significance. The rare occurrence of homozygous **2* and **3* (n= 1) and compound heterozygous patients (n =2) in our cohort did not allow us to determine a gene-dose effect.

In the setting of our study, we were able to assign 31% of the observed inter-individual variability in phenprocoumon dose to specific factors, suggesting the presence of additional unidentified environmental and/or genetic factors that modify drug response. Several comprehensive screening studies, including genome-wide association analyses, have recently been undertaken in an attempt to identify as yet unknown genetic factors influencing warfarin response [22, 39, 40]. However, insufficient evidence was obtained in all of these studies to support the inclusion of genetic variants other than *VKORC1* haplotype A/B and *CYP2C9*2* and **3* in algorithms for the prediction of coumarin maintenance dose. The only SNP found to be independently associated with warfarin dose was identified in the *CYP4F2* gene, explaining only a further 1–2% of variability [40, 41]. The pharmacogenetic effect of the *CYP4F2* V433M polymorphism was recently confirmed in acenocoumarol-treated patients [42].

There are some limitations to this study that may partially account for the rather high percentage of unexplained variability. Owing to the retrospective study design, insufficient documentation of concomitant medication interfering with phenprocoumon efficacy was available. Furthermore, no data were recorded about destabilising comorbidities, hepatic or renal dysfunctions and smoking status. Therefore, all of these potentially confounding factors could not be considered in the prediction model.

In clinical practice, the initial dose finding process for phenprocoumon is still largely empiric, and the critical issue is how closely the induction dose matches the final maintenance dose. A clinical algorithm was recently published that includes weight, age, alcohol consumption and a surgery within 1 week as the main predictors for the loading dose of phenprocoumon. This model had an explanatory power of 33% [43]. Our data endorse a fundamental role of *VKORC1* haplotypes and age and a minor role of *CYP2C9* variants in the variability of dose requirement, which is in agreement with previous reports on phenprocoumon [26, 44] and other coumarins [7, 8, 15]. With respect to more severe clinical outcomes of VKAs, such as increased bleeding risk and retarded stabilisation, the roles may be switched to a stronger association with *CYP2C9* polymorphisms [21, 23, 26]. In order to make the

Table 3 Multiple linear regression model considering the square-root transformed weekly phenprocoumon dose as dependent variable[a]

Variable	Parameter estimate	Model r^2	p value
Intercept	4.8230		<0.0001
VKORC1 −1639G>A[b]	−0.4184	0.1424	<0.0001
Age (years)	−0.0187	0.2334	<0.0001
*CYP2C9*3*[c]	−0.5535	0.2806	0.0007
*CYP2C9*2*[c]	−0.2503	0.2944	0.0622
Weight (kg)	0.0057	0.3068	0.0758

[a] √Dose=4.823–0.4148 (*VKORC1* −1639G>A) − 0.0187 (years) − 0.5535 (*CYP2C9*3*) − 0.2503 (*CYP2C9*2*) + 0.0057 (kg)

[b] Value 0 for GG, 1 for GA, 2 for AA

[c] Value 0, 1 or 2 for the number of *CYP2C9*2* and **3* alleles

√Dose square root-transformed dose

individual loading dose of phenprocoumon more predictable, genetic testing for both pharmacogenetic markers should be considered in algorithms developed in the future.

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