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Genetic determinants of acenocoumarol and phenprocoumon maintenance dose requirements

Janne Cadamuro · Benjamin Dieplinger · Thomas Felder · Igor Kedenko · Thomas Mueller · Meinhard Haltmayer · Wolfgang Patsch · Hannes Oberkofler

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Abstract

Objective The variability in warfarin dose requirement is attributable to genetic and environmental factors. Acenocoumarol (AC) and phenprocoumon (PC) are coumarin derivatives widely prescribed in European countries for the prevention and treatment of thromboembolic events. The aim of our study was to investigate the contribution of genes involved in the vitamin K cycle to AC and PC maintenance doses.

Methods Common single nucleotide polymorphisms (SNPs) in the genes encoding cytochrome P450 family member 2C9 (*CYP2C9*), vitamin K epoxide reductase complex subunit 1 (*VKORC1*), γ -glutamyl carboxylase (*GGCX*), calumenin (*CALU*) and apolipoprotein E (*APOE*) were studied in 206 patients receiving AC or PC.

Results Compared to patients with the *VKORC1* C1173C genotype, maintenance doses for AC or PC were reduced to 74.6 or 70.2% in heterozygous C1173T subjects and to 48.6 or 48.1% in homozygous T1173T subjects ($P < 0.0001$). Furthermore maintenance doses for AC and PC were significantly lower in heterozygous *CYP2C9**1*3, *CYP2C9**2*3, and in *CYP2C9**3*3 homozygote individuals compared to homozy-

gous *CYP2C9**1*1 subjects ($P = 0.0004$ and $P = 0.0017$, respectively). A multiple regression model including age, sex, last INR, *VKORC1*, and *CYP2C9* genotypes explained ~50% of the variability in AC/PC dose requirements. *CALU* genotype combinations showed minor effects on PC dose requirements. No associations with AC or PC dose requirements were observed for sequence substitutions in the *GGCX* or *APOE* genes.

Conclusion These results reveal that interindividual variability in weekly AC and PC maintenance dose requirement is mainly dependent on the *VKORC1* 1173C>T and the *CYP2C9**3 alleles. *VKORC1* and *CYP2C9* genotyping might provide helpful information to prevent serious bleeding events in subjects receiving AC or PC.

Keywords Pharmacogenetics · Single nucleotide polymorphisms · Phenprocoumon · Acenocoumarol

Abbreviations

AC	Acenocoumarol
PC	Phenprocoumon
SNP	Single nucleotide polymorphism
CYP2C9	Cytochrome P450 family member 2C9
VKORC1	Vitamin K epoxide reductase complex subunit 1
GGCX	γ -Glutamyl carboxylase
ApoE	Apolipoprotein E
RFLP	Restriction fragment length polymorphism
ANOVA	Analysis of variance

Introduction

The coumarin-based vitamin K antagonists acenocoumarol (AC) and phenprocoumon (PC) are oral anticoagulant drugs

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widely prescribed in continental European and Latin-American countries for the prophylaxis and treatment of thromboembolic events [1]. Oral anticoagulant management is challenging and requires frequent monitoring due to the marked inter- and intraindividual variability in dose requirements and the risk of serious bleeding. Although the variation in the initial and maintenance dose is known to depend on body weight, age, dietary vitamin K intake, and absorption, as well as on environmental factors such as comedication and hypermetabolic states, there is growing evidence for a strong hereditary component [2].

The highly polymorphic cytochrome P450 family member 2C9 (*CYP2C9*) is the main liver enzyme responsible for the rate limiting metabolism of AC and, at least in part, of PC [3]. Single nucleotide polymorphisms (SNPs) in the *CYP2C9* gene, including the *CYP2C9*2* and *CYP2C9*3* variants, have been shown to decrease enzymatic activity [4], have been associated with lower warfarin dose requirements [5, 6], and have been demonstrated to increase the risk for serious bleeding complications [7, 8]. Coumarin-derived drugs exert their anticoagulant effect by interfering with the recycling of reduced vitamin K [9], required as a cofactor for γ -glutamyl carboxylase (*GGCX*) [10] to activate the precursor forms of several clotting factors. Recently, a component of the vitamin K-dependent γ -carboxylation system, the vitamin K epoxide reductase complex subunit 1 (*VKORC1*), has been identified as a molecular target of coumarin-based anticoagulants [11]. Rare mutations in *VKORC1* are associated with hereditary forms of warfarin resistance [12]. In addition several SNPs, including 1173C>T (rs9934438), 1639G>A (rs9923231), and 3730G>A (rs7294), have been identified that significantly contribute to the observed variability in coumarin-based anticoagulant dose requirements [13, 14] and have been shown to increase the risk for severe bleeding complications [15].

Although these common genetic variants in the *VKORC1* and *CYP2C9* genes, together with environmental factors, account for up to 50–60% of the variance in warfarin dose requirement [2], other genes involved in the action and biotransformation of coumarin-based anticoagulants may also contribute to the variable phenotype. Associations of allelic variants with warfarin sensitivity have been described for *GGCX* [16] as well as for the chaperone calumenin [17], which binds to and inhibits *GGCX* [18]. It is also well known that a high dietary intake of vitamin K antagonizes the anticoagulant effects of coumarins [19]. Bound to chylomicrons and chylomicron remnants, lipid soluble vitamin K is cleared from the plasma through ApoE receptor-mediated endocytosis [20]. ApoE isoforms exhibit different receptor binding affinities and may therefore modulate uptake of chylomicron remnants [21]. Indeed, associations of ApoE alleles with warfarin maintenance dose requirements have been de-

scribed previously [22]. Based on numerous studies on warfarin, it is well accepted that the relationship between the prescribed dose and the individual response in oral anticoagulant therapy is determined to a considerable extent by genetic factors [23]. However our present knowledge on the implications of candidate genes and their allelic variants in the dose requirement of other frequently used coumarin-derived drugs including AC or PC is less well established. In the present study we aimed to evaluate the impact of polymorphic sequence substitutions in genes encoding for *CYP2C9*, *VKORC1*, *GGCX*, *CALU*, and *APOE* on anticoagulant therapy with AC and PC.

Materials and methods

Study population

Unrelated subjects of Austrian-German descent between 17 and 87 years of age ($n=206$) who were prescribed oral anticoagulants were consecutively recruited from April 2006 to December 2007. Information on patient's age, sex, indication for anticoagulation therapy, date of initiation of AC/PC therapy, target INR range, initial AC/PC dose, and concomitant medications as well as history of major and minor bleeding episodes was collected using a standardized questionnaire or was obtained from medical records. Major indications for oral anticoagulation treatment included thromboembolic disease, atrial fibrillation, and joint replacement (Table 1). Study participants had been under oral anticoagulation at the time of blood collection for more than 4 weeks and their individual target INR had been stabilized. The therapeutic target INR range was determined by each patient's physician and differed according to the indication for anticoagulation treatment. Interacting concomitant medications were grouped into drugs that might potentiate or attenuate the anticoagulant effect of AC and PC [24, 25]. Only patients with unchanged maintenance doses and stable INR measurements within the therapeutic range (mean target INR \pm 0.4) at four consecutive clinic visits were included in our study. All study subjects provided informed consent, and study protocols were approved by the local ethics committee.

DNA extraction and genotyping

Genomic DNA was isolated from peripheral white blood cells as described previously [26]. Real-time PCR followed by melting curve analysis was performed on the LightCycler instrument 1.0 (Roche Diagnostics, Mannheim, Germany; software version 3.5.3) using the LightCycler ApoE Mutation Detection Kit (Cat. No. 3 004 716, Roche Diagnostics) for the detection of the three common apoE

Table 1 Clinical characteristics of the study population

Variable	Acenocoumarol	Phenprocoumon	<i>P</i>
Sex (m/f)	35/45	82/44	0.034
Age (years)	65.9±14.3	59.1±12.1	0.003
Therapeutic dose (mg/week)	16.4±8.0	15.0±7.5	0.0051 ^a
Indication for treatment, <i>n</i> (%)			
Thromboembolic disease	36 (45)	35 (28)	0.0132
Atrial fibrillation	24 (30)	27 (21)	n.s.
Joint replacement	13 (16)	49 (39)	0.006
Other indication	7 (9)	15 (12)	n.s.
Concomitant medications, <i>n</i> (%)			
Potentiating medication	13 (16)	48 (38)	0.0009
Attenuating medication	6 (8)	7 (6)	n.s.
Diuretics	9 (11)	14 (11)	n.s.

Age and therapeutic dose are shown as mean ±SD

^a Adjusted for age, sex, and last INR

alleles designated $\epsilon 2$ (Cys112, Cys158), $\epsilon 3$ (Cys 112, Arg 158), and $\epsilon 4$ (Arg 112, Arg 158). For genotyping of the allelic variants *CYP2C9*2* (C430T) and *CYP2C9*3* (A1075C), the LightCycler *CYP2C9* Mutation Detection Kit (Cat. No. 3 266 982, Roche Diagnostics) was used. PCR was carried out in 20- μ l capillaries (2 μ l of sample DNA and 18 μ l of master-mix) according to the manufacturer's instructions followed by a melting curve analysis with temperature increases from 40 to 80°C (0.2°C/s) and continuous fluorescence recording. Each assay run included a reference sample for each of the three possible genotypes and a negative control.

Genotyping of the *VKORC1* 1173C>T (rs9934438) polymorphism was performed using restriction fragment length polymorphism (RFLP) detection. 5'- TCTGAACCATGTGT CAGCCAGGACC-3' and 5'-GAACAGAGAGAGGAACC-AAGGGAGTGGA-3' were used as forward and reverse primers, respectively, to amplify a 290 bp fragment in 25 μ l reactions containing 200 mM of each dNTP, 1.5 mM MgCl₂, and 0.2U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Thermocycling conditions consisted of 95°C for 5 min; followed by 45 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s; with a final extension step of 72°C for 5 min. PCR products were digested with 2 U StyI (New England Biolabs, Beverly, MA, USA) overnight at 37°C. The digested products were separated on 1% agarose gels and visualized by ethidium bromide staining and UV illumination. The accuracy of the RFLP assay was verified by dye-terminator sequencing of the respective DNA region in 25 DNA samples. The concordance of 150 RFLP-tested samples performed in duplicate was 100%, and all of the subjects enrolled in our study were successfully genotyped.

Genotyping of the *CALU* (rs2290228 and rs2307040) and *GGCX* (rs699664) single nucleotide polymorphisms was performed using predeveloped TaqMan SNP genotyping assays containing primers and probes (C_1327891_1_, C_1327902_20, and C_1036123_10; Applied Biosystems).

Allelic discrimination based on real-time PCR was carried out in 96-well PCR plates with 20 μ l final reaction volume using the iCycler iQ Multicolor Real-Time PCR Detector (Bio-Rad, Hercules, CA, USA). Reactions contained 1 μ l DNA (>150 ng/ μ l), 1 μ l primer and probe mix, 10 μ l TaqMan universal PCR Master Mix (Applied Biosystems), and 8 μ l ddH₂O. PCR conditions included initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer hybridization, and DNA synthesis at 60°C for 1 min. Typing was verified in 10% of subjects by restriction enzyme digestion.

Statistical analysis

Allele frequencies were estimated by gene counting. Agreement with Hardy-Weinberg expectations was tested using a χ^2 goodness-of-fit test. Effects of genotypes on the weekly maintenance dose were analyzed separately for AC and PC using ANCOVA adjusted for sex as well as age and last INR (treated as continuous variables). Logarithmic transformations of the weekly maintenance dose were made to meet the equal variance and normality assumptions of ANOVA. To investigate potential influences of concomitant medication, analyses were adjusted for the presence (coded as 1) or absence (coded as 0) of interacting drugs. Confounding effects by inducing or inhibiting drugs were tested separately as well as in a model adjusted for the presence/absence of both types of drugs. Multivariate regression models were used to estimate the effects of variables predicting the dose requirements of AC or PC. The weekly maintenance dose was log-transformed to fulfill general linear model assumptions and was used as dependent variable. Independent variables included sex, age, and last INR as well as *CYP2C9*2* (430C>T), *CYP2C9*3* (1075A>C), and *VKORC1* genotypes. The products of *CYP2C9*2* or *CYP2C9*3* with *VKORC1* genotypes were included in the model to determine possible

functional interactions. To determine possible interactions of genotypes with anticoagulant drugs (AC or PC), we used a regression model that contained the log-transformed weekly maintenance dose as dependent variable and age, sex, last INR anticoagulant drug (AC coded as 1 and PC coded as 2), *CYP2C9*2*, *CYP2C9*3*, or *VKORC1* genotypes, and the products of genotype and the coded drug as independent variables. This model was analyzed separately for each of the three polymorphic sites.

Results

The study cohort comprising 80 subjects receiving AC and 126 subjects treated with PC was recruited from two hemostasis clinics in Austria. Clinical characteristics of the study subjects are summarized in Table 1. The mean weekly maintenance dose was 16.4 ± 8.0 mg for AC and 15.0 ± 7.5 mg for PC, respectively. Mean age of study subjects was significantly lower, and the number of patients receiving concomitant medication known to potentiate the effects of coumarin derivatives was higher in the PC group. Genotype frequencies of SNPs in genes involved in the vitamin K cycle and in the metabolism of coumarin-derived drugs including *VKORC1* (rs9934438), *CALU* (rs2290228 and rs2307040), *GGCX* (rs699664), *APOE* (rs27412 and rs429358), and *CYP2C9* (rs1799853 and rs1057910) were determined. No deviation from the expected population genotype frequencies, as predicted by Hardy-Weinberg equilibrium, was detected for any SNP studied in our study population.

We first examined possible associations of well established SNPs in the *VKORC1* (1173C>T; rs9934438) and *CYP2C9* (430C>T; rs1799853 and 1075A>C; rs1057910) genes with AC and PC maintenance doses. Table 2 shows the mean maintenance dose requirements categorized for the different *VKORC1* and combined *CYP2C9* genotypes adjusted for age, sex, and last INR. In comparison to carriers of the *VKORC1* CC genotype, the mean AC maintenance doses were significantly reduced in a gene dose-dependent manner in CT and TT carriers ($P < 0.0001$; Table 2). A similar genotype-dose relationship was observed in the PC group ($P < 0.0001$; Table 2).

Frequencies of the three major human *CYP2C9* alleles, *CYP2C9*1* (wild-type allele), *CYP2C9*2* (430C>T), and *CYP2C9*3* (1075A>C), implicated in the metabolism of coumarin-derivates were determined. As shown in Table 2, the mean AC dose was higher in the group of *CYP2C9*1*1* homozygotes (19.74 mg/week) compared to heterozygotes with the *CYP2C9*1*3* genotype (12.56 mg/week) or the *CYP2C9*2*3* genotype (18.64 mg/week). The lowest mean maintenance dose was observed in *CYP2C9*3*3* homozygotes (6.20 mg/week). A similar relationship was

observed for PC showing lower dose requirements for heterozygote subjects with the *CYP2C9*1*3* (12.45 mg/week) and the *CYP2C9*2*3* (12.63 mg/week) genotype compared to *CYP2C9*1*1* (15.81 mg/week) wild-type individuals. In the PC group, no subjects with the *CYP2C9*3*3* genotype were present. *CYP2C9*2*2* homozygotes required the highest maintenance doses in the AC group (22.48 mg/week) and the lowest doses in the PC group (6.72 mg/week). As the number of *CYP2C9*2*2* subjects was very low in both groups ($n=3$), these results should be interpreted with caution. Indeed, no significant interactions of *CYP2C9* genotypes with the anticoagulant drug (AC or PC) were noted (data not shown). All associations remained statistically significant after consideration of concomitant medications that might have potentiating or attenuating effects (data not shown).

A multivariate regression model with log-transformed maintenance doses as the dependent variable and age, sex, last INR, and *VKORC1* and *CYP2C9* genotypes as independent variables explained ~58% of the variability in AC (Table 3) and 55% of the variability in PC dose requirements (Table 4), respectively. Furthermore no functional interaction of *VKORC1* with either of the two *CYP2C9* loci was observed (data not shown), arguing for an additive effect of the variant alleles.

To identify additional genetic modifiers of anticoagulant dose requirements, we studied possible associations of nucleotide variations in the *CALU*, *GGCX*, and *APOE* genes. As shown in Table 5, borderline significant associations were observed for genotype combinations of two SNPs (rs2290228 and rs2307040) in the *CALU* gene in the PC group only. Subjects heterozygous at both loci were characterized by the lowest maintenance dose requirements. No interactions with *VKORC1* or *CYP2C9* genotypes were observed (data not shown). We did not observe associations with dose requirements for SNPs rs28931578 and rs11542030 (*APOE*) and rs699664 (*GGCX*) in our AC or PC study groups (Table I and Table II in ESM).

Discussion

The prescription of warfarin, AC, and PC as inhibitors of the vitamin K epoxide reductase complex for long-term anticoagulation management and the prevention of thromboembolic events is growing continuously [1]. The unpredictable dose-response relationship of these drugs, frequently observed in the initial phase of the therapy, increases the risk of over-anticoagulation and resultant bleeding complications and is markedly influenced by both genetic and environmental factors [2]. Single nucleotide polymorphisms in the drug target gene *VKORC1* and the drug-metabolizing *CYP2C9* gene have been shown to account for a significant

Table 2 Relationship between *VKORC1* 1173C>T and *CYP2C9* genotype and mean acenocoumarol and/or phenprocoumon weekly dose requirement

Genotype	Acenocoumarol			Phenprocoumon		
	<i>n</i>	Average dose (SD)	<i>P</i>	<i>n</i>	Average dose (SD)	<i>P</i>
<i>VKORC1</i> C1173T						
CC	33	21.56 (7.68)		35	19.08 (7.56)	
CT	35	16.08 (7.76)		64	15.12 (4.44)	
TT	12	10.36 (5.32)	<0.0001	27	9.27 (2.82)	<0.0001
<i>CYP2C9</i>						
<i>CYP2C9</i> *1*1	44	19.74 (1.77)		86	15.81 (2.11)	
<i>CYP2C9</i> *1*2	21	16.64 (2.47)		21	12.96 (1.62)	
<i>CYP2C9</i> *2*2	3	22.48 (1.61)		3	6.72 (0.14)	
<i>CYP2C9</i> *1*3	7	12.56 (1.62)		14	12.45 (1.51)	
<i>CYP2C9</i> *3*3	3	6.20 (0.63)		0		
<i>CYP2C9</i> *2*3	2	18.64 (0.18)	<0.0004	2	12.63 (0.0)	<0.0017

Data are presented as untransformed means ± SD adjusted for age, sex, and last INR; *P* values were calculated using log-transformed maintenance dose values

proportion of the interindividual variability in anticoagulant dose requirements [27]. Most of the present knowledge on the pharmacogenetic aspects of oral anticoagulation is based on extensive studies in subjects treated with warfarin, which is predominantly prescribed in the U.S. and Northern European countries, whereas AC or PC are mainly prescribed in most other European countries. However important pharmacokinetic differences between individual coumarin-based anticoagulants are well known as AC has a very short half life (2–8 h) compared to warfarin (32–44 h) or PC (156–172 h) [28].

In the present study we confirmed previous reports showing that the interindividual variation in AC maintenance dose is related to the *VKORC1* 1173C>T genotype [15, 17, 29]. AC doses required to achieve stable therapeutic anticoagulation were significantly higher in CC wild-type subjects compared to individuals carrying the CT or TT genotype. Similar results were observed in our PC group that are in line with two recent reports [30, 31]. The 1173C>T nucleotide substitution is located in intron 1 of the *VKORC1* gene and is in complete linkage disequilibrium with a functional -1639G>A substitution located within an E-box motif in the upstream regulatory region of the *VKORC1* gene [32]. In luciferase promoter reporter assays, the -1639G allele was associated with an increased transcriptional activity when compared to the -1639A allele. Meanwhile additional functional and non-

functional *VKORC1* SNPs have been studied, and only haplotypes including the 1173C>T/-1639G>A variants have been found to segregate with high- and low-dose requirements for warfarin [13, 33].

AC, like warfarin, is mainly metabolized in the liver by *CYP2C9*, a polymorphic enzyme with a high degree of genetic variability associated with distinct activity levels [34]. In several studies, associations of two common allelic variants, *CYP2C9**2 and *CYP2C9**3, with warfarin dose requirements and with the risk of over-anticoagulation and severe bleeding episodes, especially during the initiation phase of warfarin therapy, have been observed [5, 6, 8]. Furthermore, the time required to achieve INR stability has been associated with the *CYP2C9* genotype [35]. A genotype-dose relationship and an unstable anticoagulant response have meanwhile also been reported for the *CYP2C9**3 allele in AC-treated subjects [29, 31]. We also observed that *CYP2C9**1*1 wild-type subjects required significantly higher AC doses during the maintenance phase of oral anticoagulation therapy compared to carriers of the *CYP2C9**2*3 and the *CYP2C9**3*3 genotype, whereas the contribution of the *CYP2C9**2 allele was less clear. This is consistent with previous observations showing that the *CYP2C9**2 allele was more prevalent in subjects with low-dose AC requirements, and the risk for major bleeding events was increased in individuals on AC carrying at least one *CYP2C9**2 or *CYP2C9**3 allele.

Table 3 Predictors of acenocoumarol maintenance dose requirement

Trait	Regression coefficient [standard error]	<i>P</i>
Age (years)	-0.567 [0.075]	<0.00001
Sex (f/m)	0.055 [0.077]	0.4768
Last INR	-0.082 [0.074]	0.2723
<i>VKORC1</i> (0,1,2)	-0.533 [0.075]	<0.00001
<i>CYP2C9</i> rs1799853 (0,1,2)	-0.094 [0.075]	0.2165
<i>CYP2C9</i> rs1057910 (0,1,2)	-0.428 [0.078]	<0.00001

Multiple regression analysis; *n*=80; adjusted *R*²=0.5793; genotypes were coded as 0 (homozygous wild-type), 1 (heterozygous), and 2 (homozygous mutant)

Table 4 Predictors of phenprocoumon maintenance dose requirement

Multiple regression analysis; $n=126$; adjusted $R^2=0.05514$; genotypes were coded as 0 (homozygous wild-type), 1 (heterozygous) and 2 (homozygous mutant)

Trait	Regression coefficient [standard error]	<i>P</i>
Age (years)	-0.312 [0.065]	<0.00001
Sex (f/m)	-0.022 [0.063]	0.7349
Last INR	0.062 [0.066]	0.3466
<i>VKORC1</i> (0,1,2)	-0.583 [0.064]	<0.00001
<i>CYP2C9</i> rs1799853 (0,1,2)	-0.325 [0.064]	<0.00001
<i>CYP2C9</i> rs1057910 (0,1,2)	-0.158 [0.063]	0.0137

The less important contribution of *CYP2C9* to the metabolism of PC is mainly attributed to the involvement of *CYP3A4* as an additional catalyst of PC 7-hydroxylation. Furthermore a significant portion of the drug is excreted in an unchanged form in bile and urine, while clearance of warfarin and AC occurs almost completely by their metabolism [28]. Although associations of *CYP2C9* sequence variations with reduced PC 7-hydroxylation have been shown in vitro [36] and in vivo [37], the clinical impact of *CYP2C9* variant alleles on PC dosing requirements is still controversial. Visser et al. failed to reveal significant differences in dose requirements between carriers of the *CYP2C9**2 and *CYP2C9**3 allele(s) compared to wild-type subjects in a population-based cohort from the Rotterdam Study [38]. Based on the fact that these initial studies in patients receiving PC showed no associations of variant *CYP2C9* alleles with the risk of major bleeding events, PC prescription was suggested as a clinically useful alternative for warfarin or AC in patients carrying the *CYP2C9**2 and *CYP2C9**3 alleles. Meanwhile other studies reported associations of *CYP2C9* variants with PC dose requirements and an increased risk of severe over-anticoagulation and bleeding [39, 40]. In the present study we confirmed the latter findings at least in part and observed a more pronounced effect of the *CYP2C9**2 allele on PC maintenance dose compared to the *CYP2C9**3 allele. Because of the low number of subjects harboring a single *CYP2C9**3 allele and the absence of homozygous *CYP2C9**3*3 individuals in our study group, the presented results warrant cautious interpretation and await further investigation in larger populations. Consistent with other studies, the association of the *VKORC1* 1173C>T

genotype with AC or PC dose requirements was stronger than the effects of the *CYP2C9* variants. No interactions between the *VKORC1* 1173C>T genotype and the *CYP2C9* gene variants were observed in our population arguing for an independent and additive effect of both genes.

The identification of genetic polymorphisms in the *VKORC1* and *CYP2C9* genes significantly affecting both pharmacokinetics and pharmacodynamics of coumarin-based oral anticoagulants has prompted the search for new candidate gene variants. Recently effects of genetic variants in the *CYP4F2* gene on warfarin requirement have been described [41]. Furthermore associations of SNPs in the *CALU*, the *GGCX*, and the *APOE* genes with warfarin sensitivity have been reported. Calumenin is a molecular chaperone that binds to the VKOR complex, thereby regulating the activity and warfarin sensitivity of the vitamin K-dependent γ -carboxylation system [18]. A 11C>T polymorphism (rs2290228) resulting in an Arg2Gln amino acid substitution has been described in a single patient who required a high warfarin dose, and the number of heterozygous carriers of the T allele was significantly higher in a high warfarin-dose group [42]. Recently, Wadelius et al. reported associations of additional *CALU* SNPs including an intronic 29809A>G variant (rs2307040) with warfarin dose requirements [43]. We extended these studies to subjects treated with AC or PC and report borderline significant associations of combined rs2290228 and rs2307040 genotypes with dose requirements in the PC group. In our study sample, we failed to confirm an interaction between *VKORC1* 1173C>T and the *CALU* 29809A>G variant, which has previously been reported to modulate the anticoagulant response of AC [17].

Table 5 Associations of *calumenin* genotype combinations with mean weekly acenocoumarol or phenprocoumon dose requirement

Data are presented as mean \pm SD adjusted for age, sex, and last INR

<i>Calumenin</i> genotype		Acenocoumarol			Phenprocoumon		
rs2290228 C>T	rs2307040 G>A	<i>n</i>	Average dose (SD)	<i>P</i>	<i>n</i>	<i>P</i>	
C/C	G/G	23	16.16 (5.84)		33	14.07 (4.19)	
C/C	G/A	22	16.97 (8.03)		44	15.99 (7.06)	
C/T	G/G	10	14.92 (8.68)		17	15.16 (4.89)	
C/T	G/A	10	14.03 (6.65)		13	10.47 (3.68)	
C/C	A/A	11	19.94 (11.64)		18	15.19 (7.59)	
T/T	G/A	1	11.65 (0)				
T/T	G/G	3	16.12 (6.35)	0.5565	1	27.96 (0)	0.0197

Furthermore, in contrast to recent observations in warfarin-treated Japanese subjects, we did not observe significant differences in anticoagulant dose requirement for a non-synonymous Arg325Glu polymorphism in exon 8 of the *GGCX* gene [44]. The Arg325Glu variant is located within a domain of the *GGCX* gene that mediates enzyme/substrate interactions [45]. Our study also failed to support a substantial role for allelic variants in the *APOE* gene as determinants of AC/PC maintenance doses, as has been suggested previously. Visser et al. reported slightly higher PC maintenance doses in *APOE*E4* homozygous individuals whereas carriers of the *APOE*E4* allele required lower doses of AC [46]. Inconsistent results on ApoE genotypes were also obtained in studies using warfarin [22, 43] indicating that the association between anticoagulant dose and *APOE* alleles, if any, is weak.

Our results are consistent with a strong impact of nucleotide substitutions in the *VKORC1* and *CYP2C9* genes on AC and PC anticoagulant maintenance dose, whereas genetic polymorphisms in additional components of the vitamin K cycle including *GGCX*, *CALU*, and *APOE* appear to be of negligible or minor importance. These observations are in line with a recent genomic scan arguing against the possibility that major effects on warfarin dose requirements will be found for other individual genes besides *VKORC1* or *CYP2C9* [47]. Similar results in AC-treated subjects were obtained in a recent genome-wide association study that identified the *VKORC1* and *CYP2C9* genes as the main modulators of AC dose requirements and reported minor effects of additional cytochrome P450 family members including *CYP4F2* and *CYP2C18* [48]. Nevertheless additional studies in larger populations are needed to confirm our results and to reevaluate the contribution of other genetic factors in the anticoagulant response to AC and PC. Although, as has already been suggested for warfarin, pharmacogenetic testing might substantially improve trial-and-error-based dose adjustments for AC or PC [23, 49], additional randomized clinical trials are necessary to prove the clinical usefulness of genotype-based dosing regimens for these drugs.

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