VKORC1 and *CYP2C9* Genotypes and Phenprocoumon Anticoagulation Status: Interaction Between both Genotypes Affects Dose Requirement

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In a prospective follow-up study of the effects of *VKORC1* and *CYP2C9* genotypes on the anticoagulation status of patients, we assessed the *CYP2C9* and the *VKORC1 C1173T* genotypes of patients during the initial 6 months of phenprocoumon treatment. We used linear regression models and Cox proportional hazard models to determine the effects of the *VKORC1* and *CYP2C9* genotypes on phenprocoumon dose requirements, overanticoagulation, and time to achieve stability. Allele frequencies of interest within the cohort ($N = 281$) were 40.8% *VKORC1 T-1173*, 12.8% *CYP2C9*2*, and 6.9% *CYP2C9*3*. In patients with the *VKORC1 CC* genotype, carriers of a *CYP2C9* polymorphism needed dosages that were nearly 30% lower than those for *CYP2C9*1/*1* patients ($P < 0.001$). In patients with a *VKORC1* polymorphism, differences between carriers of a *CYP2C9* polymorphism and *CYP2C9*1/*1* were far smaller and largely not statistically significant. A larger part of the variability in dose requirement was explained by the *VKORC1* genotype than by the *CYP2C9* genotype (28.7% and 7.2%, respectively). Carriers of a combination of a *CYP2C9* polymorphism and a *VKORC1* polymorphism had a strongly increased risk of severe overanticoagulation (hazard ratio (HR) 7.20, $P = 0.002$). Only carriers of a *CYP2C9*2* allele had a decreased chance to achieve stability compared to *CYP2C9*1/*1* patients (HR 0.61, $P = 0.004$). In conclusion, the *VKORC1* genotype modifies the effect of the CYP2C9 genotype on phenprocoumon dose requirements. A combination of polymorphisms of both genotypes is associated with a strongly increased risk of overanticoagulation, whereas delayed stabilization is mainly associated with the *CYP2C9* genotype.

Anticoagulants of the coumarin type are effective drugs for the treatment and prevention of thromboembolic diseases. However, these drugs have a narrow therapeutic range and show a large interindividual and intraindividual variability in dose requirement, which necessitates frequent monitoring of the anticoagulant effect and dosage adjustments. Known factors contributing to this variability are age, drug–drug interactions, ingestion of varying quantities of vitamin K, heart failure, infections, impairment of liver function,[1–5] and polymorphisms of the *CYP2C9* gene, which encodes for the main metabolizing enzyme of the coumarins.[6–10]

The presence of polymorphisms in the *VKORC1* gene has been recently identified as another source of variability in the response to coumarins. The enzyme vitamin K epoxide reductase (VKOR) reduces vitamin K 2,3-epoxide to the biologically active vitamin K hydroquinone, which catalyzes the production of the blood-clotting proteins II, VII, IX, and X by carboxylation of glutamic acid residues. Coumarins interfere with this carboxylation by inhibiting VKOR through their recently identified target protein vitamin K reductase complex subunit 1 (VKORC1), which is encoded by the homonymous gene *VKORC1*.[11,12] In several studies, an association between the presence of polymorphisms of the

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VKORC1 gene and a reduced dose requirement of warfarin,[10,13–20] acenocoumarol,[21–23] and phenprocoumon[23] has been demonstrated. Most of the studies that examined the effects of both the *VKORC1* and the *CYP2C9* genotypes showed that a larger part of the variation in dose requirement was explained by the *VKORC1* than by the *CYP2C9* genotype,[14,17,18,20–22] suggesting that the *VKORC1* genotype has a larger impact on the anticoagulation status. However, in a recent study in acenocoumarol users we demonstrated that being a carrier of a combination of variant alleles of *CYP2C9* and *VKORC1*, rather than of one variant allele, is associated with severe overanticoagulation, underlining the importance of both genotypes for the anticoagulation status.[21]

Associations between the *CYP2C9* genotype and the anticoagulation status have been convincingly demonstrated for warfarin and acenocoumarol,[6–10] carriers of a *CYP2C9*2* or *CYP2C9*3* allele requiring lower coumarin dosages and having an increased tendency to severe overanticoagulation and retarded stabilization. However, this *CYP2C9* sensitivity is less clear for phenprocoumon, a frequently used coumarin in European countries. Recently, we found an association between possession of *CYP2C9*2* or **3* polymorphisms and a lower dose requirement, severe overanticoagulation, and delayed stabilization (for the **2* allele) in a Dutch population of phenprocoumon users,[24] whereas another Dutch study did not find such associations.[25] The clinical relevance of the findings of our study has been questioned[26] because only a minor impact of the *CYP2C9* genotype on phenprocoumon metabolism has been found in pharmacokinetic studies.[27–29] Of course, it might be possible that the results of our earlier phenprocoumon study have been confounded by the at-that-time unknown *VKORC1* genotype. In order to examine the effects of both the *VKORC1* and *CYP2C9* genotypes on phenprocoumon sensitivity and their relative contributions to dose requirements, we also assessed the *VKORC1 C1173T* polymorphisms in the same cohort of outpatients of two Dutch anticoagulation clinics.

RESULTS

Of the 284 patients we analyzed in our earlier study,[24] three had no blood or DNA samples left for analysis. The remaining 281 patients were all available for analysis (**Table 1**).

Allele frequencies of *VKORC1 C-1173* and *VKORC1 T-1173* were 59.2% and 40.8%, respectively. Allele frequencies of *CYP2C9*1*, *CYP2C9*2*, and *CYP2C9*3* were 80.3%, 12.8%, and 6.9%, respectively. Allele frequencies of both genotypes were in Hardy–Weinberg equilibrium (**Table 1**).

For combined *VKORC1* and *CYP2C9* genotypes, numbers of patients within each combination of genotypes, mean weekly doses, numbers of patients with severe overanticoagulation, numbers of patients in whom stability was achieved, and number of days until stability was achieved are summarized in **Table 2**.

The *CYP2C9* and *VKORC1* genotypes modified each other's effects on dose requirements. In our regression models, we found a statistical interaction between the

Table 1 Characteristics of patients taking phenprocoumon treated by two anticoagulation clinics (*N*=281)

	CYP2C9*1/*1[a] (*n*=183 (65.1%))	CYP2C9*2[a] (*n*=61 (21.7%))	CYP2C9*3[a] (*n*=37 (13.2%))	VKOR CC[b] (*n*=106 (37.7%))	VKOR CT[b] (*n*=121 (43.1%))	VKOR TT[b] (*n*=54 (19.2%))
Men (no. (%))	101 (55.2)	36 (59.0)	19 (51.4)	56 (52.8)	70 (57.9)	30 (55.6)
Age (years) (mean (SD))	64.3 (15.8)	65.6 (16.1)	66.0 (15.0)	63.9 (15.0)	64.3 (15.8)	67.9 (16.7)
Maximum follow-up time (days) (mean (SD))	151 (35)	148 (40)	155 (34)	146 (41)	151 (35)	161 (24)
Number of INR measurements (mean (SD))	13.8 (3.8)	14.1 (3.9)	13.8 (3.7)	13.7 (3.7)	13.6 (3.8)	14.8 (3.9)
Indication for acenocoumarol treatment (no. (%))						
Atrial fibrillation	93 (50.8)	37 (60.7)	21 (56.8)	52 (49.1)	66 (54.5)	33 (61.1)
Deep vein thrombosis	43 (23.5)	11 (18.0)	4 (10.8)	21 (19.1)	27 (22.3)	10 (18.5)
Pulmonary embolus	37 (20.2)	11 (18.0)	8 (21.6)	26 (24.5)	21 (17.4)	9 (16.7)
Other indications	10 (5.5)	2 (3.3)	4 (10.8)	7 (6.6)	7 (5.8)	2 (3.7)
Relevant comedication during follow-up (no. (%))						
NSAIDs[c]	12 (6.6)	4 (6.6)	8 (21.6)	8 (7.5)	11 (9.1)	5 (9.3)
Antibiotics	34 (18.6)	12 (19.7)	11 (29.7)	20 (18.9)	27 (22.3)	10 (18.5)
Patients with congestive heart failure (no. (%))	18 (9.8)	4 (6.6)	4 (10.8)	9 (8.5)	12 (9.9)	5 (9.3)

INR, international normalized ratio; NSAIDs, nonsteroidal anti-inflammatory drugs. [a]Full details of *CYP2C9*-genotype are as follows: *CYP2C9*1/*1*, *n*=183 (65.1%); *CYP2C9*1/*2*, *n*=56 (19.9%); *CYP2C9*1/*3*, *n*=29 (10.3%); *CYP2C9*2/*2*, *n*=5 (1.8%); *CYP2C9*2/*3*, *n*=6 (2.1%); *CYP2C9*3/*3*, *n*=2 (0.7%). Allele frequencies are as follows: *1, 80.3%; *2, 12.8%; *3, 6.9% (Hardy–Weinberg χ^2=0.93, *P*=0.82). [b]Allele frequencies are as follows: *C-1173* allele, 59.2%, *T-1173* allele, 40.8% (Hardy–Weinberg χ^2=3.29, *P*=0.070). [c]Only known CYP2C9 substrates have been included (in this cohort celecoxib, diclofenac, ibuprofen, meloxicam, and naproxen).[35]

Table 2 Phenprocoumon mean weekly doses, overanticoagulation (INR > 6.0), days until stabilization and stability for combinations of *VKORC1* and *CYP2C9* genotypes

Combined VKORC1-CYP2C9 Genotype	*n*	Dose (mean, 95% CI) (mg/week)	INR > 6.0 (No. (%))	Patients with stabilization (no. (%))	Days until stabilization (mean (SD))
*CC-*1/*1*	63	22.4 (20.4–24.3)	3 (4.8)	55 (87.3)	71 (39)
*CC-*2*	27	15.4 (13.5–17.4)	4 (14.8)	19 (70.4)	81 (43)
**1/*2*	26	15.3 (13.3–17.3)	4 (15.4)	18 (69.2)	83 (44)
**2/*2*	1	17.0	0	1 (100)	52
*CC-*3*	16	15.6 (13.2–17.9)	4 (25.0)	11 (68.8)	51 (31)
**1/*3*	11	16.8 (13.6–20.0)	3 (27.3)	7 (63.6)	45 (26)
**2/*3*	4	12.7 (6.2–19.1)	1 (25.0)	3 (75.0)	69 (45)
**3/*3*	1	15.8	0	1 (100)	42
*CT-*1/*1*	95	16.4 (15.4–17.3)	12 (12.6)	83 (87.4)	62 (33)
*CT-*2*	16	15.0 (11.3–18.8)	5 (31.3)	9 (56.3)	78 (39)
**1/*2*	14	14.1 (10.6–17.6)	5 (35.7)	8 (57.1)	79 (41)
**2/*2*	2	22.5	0	1 (50.0)	74
*CT-*3*	10	14.4 (12.4–16.6)	0	8 (80.0)	49 (11)
**1/*3*	9	14.3 (11.6–17.0)	0	7 (77.8)	48 (12)
**2/*3*	1	14.7	0	1 (100)	56
*TT-*1/*1*	25	10.6 (9.1–12.2)	2 (8.0)	22 (88.0)	68 (40)
*TT-*2*	18	9.4 (7.0–11.8)	7 (38.9)	16 (88.9)	82 (41)
**1/*2*	16	9.8 (7.2–12.5)	6 (37.5)	14 (87.5)	78 (38)
**2/*2*	2	6.5 (−12.2–25.2)	1 (50.0)	2 (100)	104 (72)
*TT-*3*	11	8.5 (6.4–10.6)	4 (36.4)	11 (100)	63 (34)
**1/*3*	9	8.1 (5.8–10.5)	4 (44.4)	9 (100)	69 (35)
**2/*3*	1	13.2	0	1 (100)	31
**3/*3*	1	7.4	0	1 (100)	43

SD, standard deviation; CI, confidence interval.

CYP2C9 and *VKORC1* genotypes, *P*-values for two of the product terms in our regression model being lower than 0.05. In carriers of a *CYP2C9*2* or *CYP2C9*3* allele with the *VKORC1 CC* genotype, the weekly dosages were considerably and significantly lower than in *VKORC1 CC–CYP2C9*1/*1* patients (point estimates of the percentages of dose reduction compared to *CYP2C9*1/*1*: 27.7% for *CYP2C9*2* and 28.1% for *CYP2C9*3*). However, if patients had also the *VKORC1 CT* or *VKORC1 TT* genotype was the differences between carriers of a *CYP2C9*2* or *CYP2C9*3* allele and *CYP2C9*1/*1* patients were far smaller and generally not statistically significant. Only in patients with the *VKORC1 TT* genotype was the difference between *CYP2C9*1/*1* and *CYP2C9*3* patients marginally significant (**Table 3**). The combination of *VKORC1* genotype, *CYP2C9* genotype, interaction between both genotypes, age, and sex explained 54.7% of the variation in mean weekly dosage, adjusted mean R^2 being 28.7% for *VKORC1* genotype, 14.1% for age, 7.2% for *CYP2C9* genotype, 1.6% for interaction between *VKORC1* and *CYP2C9* genotypes, and 0.8% for sex.

Analysis of the mean dose during the entire follow-up period from the 30th day after entry as a measure for dose requirement did not result in other insights. The explained percentage of variation in mean weekly dosage as well as the adjusted dosage differences between combined genotypes were fully comparable with the differences we

Table 3 Dose differences in phenprocoumon for combined *VKORC1* and *CYP2C9* genotypes[a,b] (raw differences are not shown, because they were very similar to the adjusted differences)

Genotype[c]	*N*	Adjusted difference (mean, 95% CI)[d]	Adjusted difference (%)
*VKOR CC – CYP2C9*1/*1*	55	22.4 mg/week (Reference)	Ref
*VKOR CC – CYP2C9*2*	19	−6.2 (−8.6 to −3.9)	−27.7
*VKOR CC – CYP2C9*3*	11	−6.3 (−9.1 to −3.4)	−28.1
*VKOR CT – CYP2C9*1/*1*	83	−6.0 (−7.6 to −4.5)	−26.8
*VKOR CT – CYP2C9*2*	9	−7.4 (−10.6 to −4.3)	−33.0
*VKOR CT – CYP2C9*3*	8	−7.7 (−11.0 to −4.4)	−34.3
*VKOR TT – CYP2C9*1/*1*	22	−11.1 (−13.3 to −8.9)	−49.6
*VKOR TT – CYP2C9*2*	16	−12.4 (−15.0 to −10.0)	−55.4
*VKOR TT – CYP2C9*3*	11	−13.6 (−16.5 to −10.8)	−60.7

[a]Mean dose (in mg/week) of stabilized patients (if stability was not achieved, no mean dose was computed). [b]*P*-values for interaction between genotypes: *VKORC1 CT* × *CYP2C9*2*, 0.012; *VKORC1 CT* × *CYP2C9*3*, 0.059; *VKORC1 TT* × *CYP2C9*2*, 0.008; and *VKORC1 TT* × *CYP2C9*3*, 0.062, in which *VKORC1 CT*, *VKORC1 TT*, *CYP2C9*2*, and *CYP2C9*3* are dummies in the linear regression equation: mean phenprocoumon dose=a+b.*VKORC1 CT*+c.*VKORC1 TT*+d.*CYP2C9*2*+e.*CYP2C9*3* which was used to assess an interaction between the *VKORC1* and *CYP2C9* genotypes, and in which a is the intercept and b–e are coefficients of the separate factors in the equation. [c]*P*-values for other comparisons: within *CYP2C9*2* stratum difference between *VKORC1 CC* and *VKORC1 CT*, *P*=0.41, difference between *VKORC1 CC* and *VKORC1 TT*, $P<0.001$. Within *CYP2C9*3* stratum difference between *VKORC1 CC* and *VKORC1 CT*, *P*=0.21, difference between *VKORC1 CC* and *VKORC1 TT*, $P<0.001$. Within *VKORC1 CT* stratum there were no significant differences between *CYP2C9*1/*1* and *CYP2C9*2*, *P*=0.42 or *CYP2C9*3*, *P*=0.22. Within *VKORC1 TT* stratum difference between *CYP2C9*1/*1* and *CYP2C9*2*, *P*=0.53 and difference between *CYP2C9*1/*1* and *CYP2C9*3*, *P*=0.036. [d]All found differences were statistically significant ($P<0.001$). Dose requirements were adjusted for differences in heart failure, sex and age.

found for stabilized patients and statistical significance was maintained for all found differences and for the product terms between the *CYP2C9* and *VKORC1* genotypes (data not shown).

For the outcome severe overanticoagulation, we found no statistical interaction between the *VKORC1* and *CYP2C9* genotypes. After adjustment for potential confounders, including *VKORC1* genotype, carriers of a *CYP2C9*2* or **3* allele had a significantly increased risk of severe overanticoagulation compared to subjects with the *CYP2C9*1/*1* wild type. If both alleles were considered separately, the risk was only significantly increased in *CYP2C9*2* carriers ($P=0.001$), whereas there was a strong trend in *CYP2C9*3* carriers ($P=0.060$). Patients with the *VKORC1 TT* genotype had a significantly increased risk of severe overanticoagulation compared to *VKORC1 CC* patients, whereas this risk was not significantly increased in patients with the *VKORC1 CT* genotype (**Table 4**). If combined *VKORC1–CYP2C9* genotypes were considered, the risk for severe overanticoagulation was most strongly increased in patients with a combination of *CYP2C9*2* or *CYP2C9*3* with either *VKORC1 CT* or *VKORC1 TT* compared to patients with no polymorphism of *VKORC1* or *CYP2C9* (*VKORC1 CC–CYP2C9*1/*1*) (**Table 4**, **Figure 1**).

We also found no statistical interaction between the *VKORC1* and *CYP2C9* genotypes for time to achieve stability. Within the *CYP2C9* genotype, we found differences in time to achieve stability; within the *VKORC1* genotype, we did not find any differences (**Figure 2**). In patients with a *CYP2C9*2* allele, the chance to achieve stability within the follow-up period was significantly decreased compared to *CYP2C9*1/*1* patients (adjusted HR 0.61, 95% CI 0.43–0.86, $P=0.004$), whereas we found no changed risk for *CYP2C9*3* patients (adjusted HR 1.01, 95% CI 0.68–1.49, $P=0.98$) (**Figure 2a**).

Table 4 Association between severe overanticoagulation (INR > 6.0) and *CYP2C9* and *VKORC1* genotypes and combined *CYP2C9* and *VKORC1* genotype (raw differences are not shown because they were very similar to the adjusted differences)

Genotype	Adjusted HR (95% CI)	*P*-value
Separate genotypes		
*CYP2C9*1/*1*	1 (reference)	
*CYP2C9*2* or **3*	3.02 (1.62–5.65)[b]	0.001[a]
CYP2C9*2	3.37 (1.68–6.75)[b]	0.001[a]
*CYP2C9*3*	2.26 (0.96–5.30)[b]	0.060
VKORC1 CC	1 (reference)	
VKORC1 CT or *TT*	1.92 (0.96–3.83)[c]	0.067
VKORC1 CT	1.69 (0.79–3.64)[c]	0.18
VKORC1 TT	2.28 (1.02–5.10)[c]	0.045[a]
Combined genotypes		
*VKOR CC – CYP2C9*1/*1*	1 (reference)	
*VKOR CC– CYP2C9*2* or **3*	4.56 (1.20–17.3)[d]	0.026[a]
VKOR CT or *TT – CYP2C9*1/*1*	2.72 (0.78–9.49)[d]	0.12
VKOR CT or *TT – CYP2C9*2* or **3*	7.20 (2.10–24.7)[d]	0.002[a]

HR, hazard ratio; CI, confidence interval. [a]Statistically significant difference ($P<0.05$). [b]Adjusted for differences in *VKORC1* genotype, heart failure, sex, and age. [c]Adjusted for differences in *CYP2C9* genotype, heart failure, sex, and age. [d]Adjusted for differences in heart failure, sex, and age.

In patients with the *VKORC1 CT* or *VKORC1 TT* genotype, the chance to achieve stability was not significantly different from *VKORC1 CC* patients (adjusted HR 1.01, 95% CI 0.75–1.36, $P=0.95$ for *VKORC1 CT* and 1.16, 95% CI 0.81–1.68, $P=0.42$ for *VKORC1 TT*) (**Figure 2b**).

Analysis of our data after allocating patients with the CYP2C9*2/*3 genotype to the CYP2C9*2 group (instead of the CYP2C9*3 group) did not change our results (data not shown).

Analysis of our data with a separate group of homozygous carriers of two CYP2C9 variant alleles (CYP2C9*2/2, CYP2C9*2/*3, and CYP2C9*3/*3) also did not change our results. Point estimates for homozygous carriers were generally similar to those for the heterozygous carriers of a wild type and a variant allele, in most cases with loss of clinical significance as a consequence of low numbers in this group.

Exclusion of all users of nonsteroidal anti-inflammatory drugs and antibiotics did not change our results considerably. The outcomes for dosage and time to achieve stability remained the same, without any loss of significance. For the outcome severe overanticoagulation, significance for the risk in carriers of the *CYP2C9*2* allele compared to *CYP2C9*1/*1* wild-type patients was lost (HR 2.36, $P = 0.072$), whereas significance was just achieved for the risk in carriers of a *CYP2C9*3* allele (HR 3.46, $P = 0.016$). For the combined *VKORC1–CYP2C9* genotypes, significance for the increased risk in *VKORC1 CC–CYP2C9*2* or *3 patients compared to *VKORC1 CC–CYP2C9*1/*1* patients was lost (HR 2.70, $P = 0.20$), but the risk in patients with a *VKORC1* and a *CYP2C9* polymorphism was even more strongly increased than in our main cohort (HR 8.68, $P = 0.005$) (further data not shown).

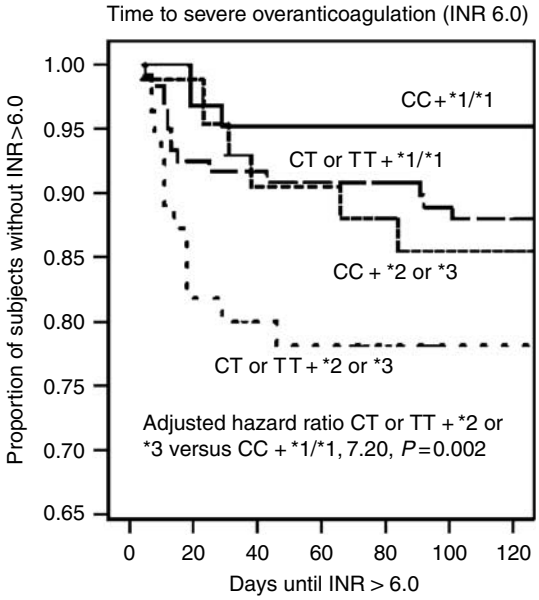

Figure 1 Kaplan–Meier survival curves for time to severe overanticoagulation. Hazard ratio (HR) adjusted for differences in age, sex and heart failure. *CC*, *CT*, and *TT* are the genotypes of VKORC1, *1/*1, *2, and *3 are the genotypes of CYP2C9 (see the text). INR, international normalized ratio.

DISCUSSION

The results of our study, in which we evaluated the effects of *VKORC1* as well as *CYP2C9* genotypes on the anticoagulation status of patients taking phenprocoumon, strongly suggest that the association between possession of a variant *CYP2C9* allele and a decreased mean weekly dose requirement we found in our earlier study within this cohort[24] is modified by the *VKORC1* genotype.

The allele frequencies we found for the *C-1173* allele (59.25%) and the *T-1173* allele (40.75%) were in accordance with other studies in several populations, *T-1173* allele frequencies varying from 39.1% to 45.8%.[15,21,22,30]

Concerning the lower maintenance dose in carriers of a *VKORC1* polymorphism, our findings are in good agreement with the study of Reitsma *et al.*,[23] the only other study that examined the association between *VKORC1 C1173T* genotype and phenprocoumon anticoagulation. Their main

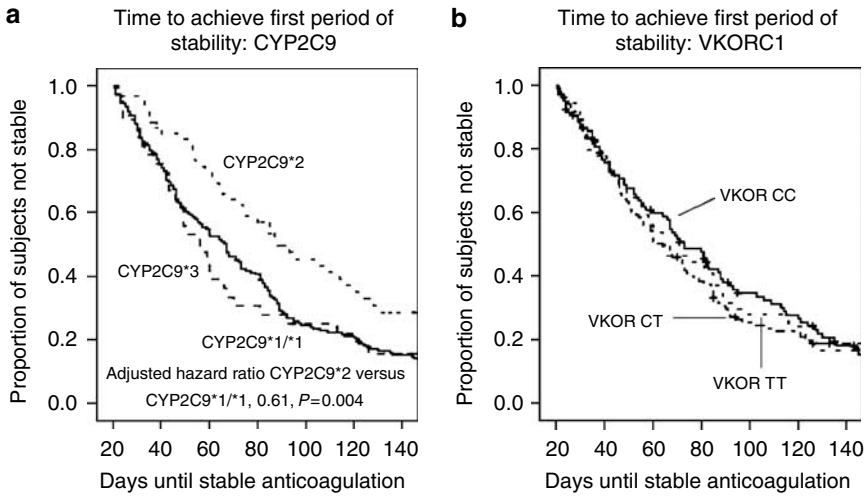

Figure 2 Kaplan–Meier curves for time to achieve period of stability, plotted for (**a**) the CYP2C9 genotype and (**b**) the VKORC1 genotype. **a** HR adjusted for differences in age, sex, heart failure, and VKORC1 genotype. **b** There were no significant differences between VKORC1 *CC*, *CT*, and *TT*. *CT* versus *CC*, HR 1.01, $P = 0.95$. *TT* versus *CC*, HR 1.16, $P = 0.42$.

findings were an increased risk of bleeding and decreased dose requirement in carriers of at least one *T* allele compared to *VKORC1 CC* patients. In contrast to our study, Reitsma *et al.*[23] did not take the *CYP2C9* genotype into account. However, it is possible to compare our results with those of Reitsma *et al.*[23] if we calculate the weighted mean of the daily phenprocoumon dosages of patients with the *VKORC1 CC*, *VKORC1 CT*, and *VKORC1 TT* genotypes, adjusting for the frequencies of the *CYP2C9* genotypes. These recalculations result in the following dose requirements in stabilized patients: *VKORC1 CC* (2.9 mg/day) (Reitsma *et al.*[23] (2.9 mg/day)), *VKORC1 CT* (2.3 mg/day) (Reitsma *et al.*[23] (2.6 mg/day)), *VKORC1 TT* (1.5 mg/day) (Reitsma *et al.*[23] (1.4 mg/day)). Our recalculated mean dose requirements for the different *VKORC1* genotypes are in remarkable agreement with those of Reitsma *et al.*,[23] corroborating a decreased phenprocoumon dose requirement in patients with a *VKORC1* polymorphism compared to *VKORC1* wild-type patients.

Compared to *CYP2C9* wild-type patients, carriers of a *CYP2C9*2* or **3* allele needed considerably lower doses if they also had the *VKORC1 CC* genotype. However, if patients had the *VKORC1 CT* or *TT* genotype, differences in dose requirement between *CYP2C9* wild-type patients and carriers of a *CYP2C9*2* or **3* allele were much smaller and not in all cases statistically significant. In fact, the *CYP2C9*1/*1*→*CYP2C9*2* or **3* shift within the *VKORC1 CC* stratum has about the same impact as the *VKORC1 CC*→*VKORC1 CT* shift in general (**Table 3**). The finding that the impact of being a carrier of a variant allele of *CYP2C9* on phenprocoumon dose requirement changes with the *VKORC1* genotype has not been reported in similar studies with the other coumarins warfarin[13–20] and acenocoumarol.[21,22] A possible explanation for this remarkable effect modification could be that the sensitivity to the S- and R-enantiomers of phenprocoumon changes with the *VKORC1* genotype. Like the other coumarins, phenprocoumon is a racemic mixture of a S- and a R-enantiomer, S-phenprocoumon being biologically more active (1.6 to 2.6 ×) than R-phenprocoumon.[31] The impact of the *CYP2C9* genotype on clearance is far greater for S-phenprocoumon than for R-phenprocoumon. Although *CYP2C9* polymorphisms reduce the metabolism of S-phenprocoumon considerably, the overall metabolism of (both S- and R-) phenprocoumon depends less on the *CYP2C9* genotype than the overall metabolism of acenocoumarol or warfarin.[27,29] So, the more S-phenprocoumon contributes to the overall anticoagulant activity, the greater the role of the *CYP2C9* genotype. It is possible that S- and R-phenprocoumon display larger differences in anticoagulant activity for the VKORC1 target protein produced by *VKORC1* wild-type patients than for the VKORC1 target protein produced by *VKORC1 CT* or *TT* patients. We are aware that this explanation is speculative and has to be tested in pharmacodynamic studies. Of course, we also have to consider the possibility that the effect modification we found was a chance finding, because our results are derived from a reanalysis of a data set in which we did not define *a priori* that we wanted to test for an interaction between genotypes. So, confirmation of our findings in an independent data set is warranted.

Few studies have examined the association between the phenprocoumon anticoagulation status and the *CYP2C9* genotype. The pharmacokinetic studies by Kirchheiner *et al.*[29] and Ufer *et al.*[27] were conducted in healthy volunteers and demonstrated a more limited role for the enzyme CYP2C9 in the overall elimination of S- and R-phenprocoumon than in the elimination of warfarin and acenocoumarol. However, their finding that the elimination of S-phenprocoumon is considerably reduced in carriers of *CYP2C9* polymorphism supports the significant dose differences we found between carriers of a *CYP2C9*2 or *3* allele and *CYP2C9*1/*1* patients with the *VKORC1 CC* genotype. In two studies of Hummers-Pradier *et al.*[32] and Visser *et al.*,[25] no significant differences in dose requirements between patients with different *CYP2C9* genotypes were found. In contrast, we found significant differences in dose requirements between carriers of a *CYP2C9*2* or **3* allele and *CYP2C9* wild-type patients in our earlier study, which was conducted within the same population as this one.[24] Our results strongly suggest that the contribution to differences between *CYP2C9* genotypes is mainly provided by patients with the *VKORC1 CC* genotype. In Caucasian populations, the percentage of patients who have the *VKORC1 CC* genotype is smaller than 40% (in our study 37.7%), which means that overall differences between *CYP2C9* genotypes will only be found in large populations if the *VKORC1* genotype is not taken into account. As a consequence, the larger number of patients in our study compared to the studies of Visser *et al.*[25] and Hummers-Pradier *et al.*[32] seems a plausible explanation for the apparent discrepancies between our study and theirs. The explained variability in dose requirement by the combination of *VKORC1*, *CYP2C9* genotype, age, and several other factors is in accordance with other studies in users of warfarin,[16,18,22] as is our finding that the *VKORC1* genotype explains a larger part of the dose variability than the *CYP2C9* genotype.[17,18,21,22] In contrast with a study we recently conducted in acenocoumarol users,[21] we found no modification of the association between the *CYP2C9* genotype and severe over-anticoagulation by the *VKORC1* genotype. The risks of having *CYP2C9* and *VKORC1* variant alleles seem to be additive rather than multiplicative. This indicates that coumarins differ in their sensitivities to combinations of *VKORC1* and *CYP2C9* genotypes, which is in itself not surprising in view of the earlier noticed differences in CYP2C9 sensitivities.

In this study, we only found an association between being a carrier of the *CYP2C9*2* allele and a decreased chance to achieve stability compared with *CYP2C9*1/*1* subjects. This indicates that the process of finding the right dose requirement is most difficult in *CYP2C9*2* carriers. That the search for a stable phenprocoumon dose regimen is more associated with the *CYP2C9* genotype than with the *VKORC1*

genotype is in agreement with our findings in another study we recently conducted in acenocoumarol users.[21]

Some limitations of our study have to be considered. Because we only had medical data from anticoagulation clinics, we could have missed relevant data about comorbidities and comedication. However, we were able to exclude subjects with potentially destabilizing hepatic dysfunction and thyroid disease from entry in our study, which has enhanced the homogeneity of our cohort. A second limitation is the lack of knowledge about the duration of potentially confounding comedication use. However, we were able to eliminate users of CYP2C9 inhibiting drugs from analysis. Moreover, reanalysis of all outcomes after exclusion of all incident users of antibiotics and NSAIDs during follow-up did not result in essentially other outcomes.

In conclusion, our study shows that in phenprocoumon users the differences in dose requirements between patients with different *CYP2C9* genotypes are modified by the *VKORC1* genotype, that differences between carriers of a *CYP2C9*2* or **3* allele and patients with the *CYP2C9*1/*1* genotype are mainly relevant in *VKORC1 CC* patients and that the *VKORC1* genotype explains a larger part of the dose variability than the *CYP2C9* genotype. Overanticoagulation is most strongly associated with possession of polymorphisms of *VKORC1* and *CYP2C9*, whereas time to achieve stability is only associated with the *CYP2C9* genotype. These results suggest that preceding knowledge of both *VKORC1* and *CYP2C9* genotypes could contribute to a safer treatment with phenprocoumon.

METHODS

Study design and patients. This study was conducted in the same cohort of phenprocoumon-using outpatients in whom we earlier examined the association between *CYP2C9* genotype and anticoagulation status. For full details not described in this article, we refer to the report of our study previously published in this journal.[24]

In brief, the original study was a prospective follow-up study at two Dutch anticoagulation clinics in patients who started phenprocoumon therapy between October 2002 and July 2003. Exclusion criteria were hepatic dysfunction, thyroid disease, and use of pharmacokinetically interacting drugs at the start of phenprocoumon therapy. These data were retrieved from the medical files of the anticoagulation clinics. Pharmacokinetically interacting drugs were inhibitors of CYP2C9 like gemfibrozile, strong inhibitors of CYP3A4 like itraconazole, and inducers of liver enzymes like carbamazepine. They were identified by means of the Dutch Standard Management Coumarin Interactions, which is used as a reference by all Dutch anticoagulation clinics and pharmacies[33] and can also be found in the review by Harder and Thurmann.[34] The Medical Ethical Committee of the Leiden University Medical Centre approved our study.[24]

Data collection and follow-up period. We collected patients' characteristics and clinical data as recorded by the anticoagulation clinics in a database. The weekly dosage for each patient was assessed from the dose schemes. For *VKORC1* genotyping, we used the samples in which we assessed the *CYP2C9* genotypes in our earlier study. The *VKORC1 C1173T* genotype was assessed in intron 1 of the *VKORC1* gene. This polymorphism appears to be as informative about warfarin sensitivity as five *VKORC1* haplotypes, which are predictive for the warfarin dose requirement and which together account for 96–99% of the total haplotypes in European-American Caucasian populations.[17]

Patients were followed up from the first date of phenprocoumon use (entry date) until the end of the observation period of maximally 180 days.

Genotyping. For CYP2C9 genotyping, we refer to our previous study.[24] A LightCycler (Roche Diagnostics, Mannheim, Germany) assay was used for the detection of the *VKORC1 C1173T* polymorphism. During the melting curve analysis, the hybridization probes dissociate from the target DNA at specific melting temperatures. The presence of a C-allele introduces a destabilizing mismatch with the fluorescenting probes, which results in a decreased melting temperature. During setup, all LightCycler analyses ($n = 25$, nine *CC*, 11 *CT*, and five *TT*) were compared with restriction fragment length polymorphism. Both methods of analysis were checked by testing externally obtained patient samples of known *VKORC1 C1173T* (*CC*, *CT*, and *TT*) genotype (courtesy of Wadelius *et al.*, Uppsala). The genotypes of the provided samples were established with minisequencing based on primer oligo base extension and matrix-assisted laser desorption/ionization–time of flight mass spectrometry. Comparison between LightCycler genotyping and restriction fragment length polymorphism samples showed completely concordant results.[21]

Assessment of the presence or absence of the single-nucleotide polymorphism *C1173T* results in three different genotypes: *VKORC1 CC* (wild type), *VKORC1 CT*, and *VKORC1 TT*.

Outcomes. The outcomes that were chosen to establish the effects of both *CYP2C9* and *VKORC1* genotypes on three representative parameters of coumarin sensitivity were:

1. Mean weekly phenprocoumon dosage during the first period of stability.
2. Severe overanticoagulation (defined as INR > 6.0) during the observation period. An INR > 6.0 is associated with a considerably increased major bleeding risk.[35,36]
3. The time to achieve a first period of stability, which was calculated as the time (in days from the entry date) until the first of three consecutive INR measurements within the therapeutic range, with a maximum difference between the mean daily dosages of 10%.

Calculations and statistical analysis. For assessment of deviations of allele frequencies from Hardy–Weinberg equilibrium, we used the χ^2 test.

For comparisons between genotypes, patients were divided into three categories for both *CYP2C9* and *VKORC1*. For the *CYP2C9* genotype, homozygous carriers of the *CYP2C9* wild-type allele (*CYP2C9*1/*1*) formed the reference group; the other two groups consisted of carriers of the *CYP2C9*2* and *CYP2C9*3* alleles. Because of the low prevalence of subjects carrying two allelic variants, heterozygous and homozygous subjects were included in the same genotype category. *CYP2C9*2/*3* subjects were allocated to the *CYP2C9*3* group, but we also analyzed our outcomes after allocating **3* subjects to the *CYP2C9*2* group. Moreover, we reanalyzed all our outcomes after having made a separate category of homozygous carriers of *CYP2C9* allelic variants (*CYP2C9*2/*2, CYP2C9*2/*3*, and *CYP2C9*3/*3*). For the *VKORC1 C1173T* genotype, homozygous carriers of the *VKORC1* wild-type allele (*VKORC1 CC*) formed the reference group, the other two groups consisted of patients with the *VKORC1 CT* and the *VKORC1 TT* genotype.

When we found an interaction between the *CYP2C9* and *VKORC1* genotypes, we compared all combined *CYP2C9–VKORC1* genotypes with patients who were homozygous carriers of the

wild-type allele of both genotypes (*VKORC1 CC–CYP2C9*1/*1*) as a reference.

Mean dose requirements were calculated for patients who achieved stability during the follow-up period, because dose requirements in nonstabilized patients are, by definition, less certain and possibly not representative for the definitive dose requirements after stabilization. To assess whether our results changed considerably if we included all patients, we compared the dose requirements in stabilized patients with the mean dose requirement in all patients from the 30th day after the entry date until the end of the follow-up period.

To assess differences in mean weekly phenprocoumon dosages during the first period of stability and percentage of variability explained by *VKORC1* and *CYP2C9* genotypes, we used linear regression modeling.

To compare our phenprocoumon dosages with the study that did not take the CYP2C9 genotype into account, we recalculated a weighted mean for patients with the *VKORC1 CC*, *VKORC1 CT*, and *VKORC1 TT* genotypes by means of the following equation:

$$\begin{aligned}\text{Dose } VKORC1\ XX = &(D_{\text{VKORC1 XX}-\text{CYP2C9*1/*1}} \\ &\times N_{\text{CYP2C9*1/*1}}/N_{\text{VKORC1 XX}}) \\ &+ (D_{\text{VKORC1 XX}-\text{CYP2C9*2}} \\ &\times N_{\text{CYP2C9*2}}/N_{\text{VKORC1 XX}}) \\ &+ (D_{\text{VKORC1 XX}-\text{CYP2C9*3}} \\ &\times N_{\text{CYP2C9*3}}/N_{\text{VKORC1 XX*}})\end{aligned}$$

in which XX is *CC*, *CT*, or *TT*, *D* is the dose, and *N* is the number of patients within the *VKORC1 XX* stratum.

We also determined the contributions of the *CYP2C9* and *VKORC1* genotypes, age, and sex to the phenprocoumon dose requirements, for which we used a linear regression model with these factors and with product terms between the *VKORC1* and *CYP2C9* genotypes. The adjusted mean R^2 value of the model explained the variability of the mean weekly dosage. Partial R^2 values for each of the contributing factors were assessed by a backward selection procedure.

To assess HRs of severe overanticoagulation and time to achieve stability, we used Cox proportional hazard modeling.

We examined effect modification by introducing product terms in our models between the *VKORC1* and *CYP2C9* genotypes and between each of these genotypes and other factors such as sex and age. In all models, we adjusted for the potential confounders age and sex and the comorbidity heart failure, which has been identified as an independent risk factor for severe overanticoagulation.[37]

To adjust for confounding comedication, the best strategy would have been to include potential interacting drugs as time-varying covariates in our models. However, files from anticoagulation clinics from which we retrieved our data did not provide reliable information on duration of comedication use. Therefore, we excluded all subjects who started using CYP2C9 inhibiting drugs during the follow-up time from our analyses.[33,34]

Moreover, we tested the robustness of our findings by reanalyzing our outcomes after exclusion of those patients who used nonsteroidal anti-inflammatory drugs which are known CYP2C9 substrates and antibiotics, which can both contribute to over-anticoagulation and instability.[3,38]

Statistical analyses were performed with the statistical software package SPSS 12 (version 12.0; SPSS, Chicago, IL).

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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