

The *c.*-1639G > A polymorphism of the *VKORC1* gene is a major determinant of the response to acenocoumarol in anticoagulated patients

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Summary

Much of the variability in the sensitivity to warfarin in anticoagulated patients is associated with the *c.*-1639G > A polymorphism of the *vitamin K-epoxide reductase (VKORC1)* gene. However, its association with the acenocoumarol dose in patients under anticoagulant therapy has not been studied. The *c.*-1639G > A genotype of *VKORC1* was determined in 113 patients on stable anticoagulation requiring low ($n = 42$), medium ($n = 42$) or high ($n = 21$) acenocoumarol doses. To evaluate the association between acenocoumarol requirements and the *c.*-1639G > A variant, multivariate logistic regression models were fitted, adjusting for age, gender, and the *c.*430C > T and *c.*1075A > C variants of *cytochrome P450 2C9 (CYP2C9)*. A total of 90.5% of the patients in the low acenocoumarol dose group carried the A allele of *VKORC1:c.*-1639G > A. The A allele independently increased the odds of requiring a low acenocoumarol dose [odds ratio (OR) 9.4; 95% confidence interval (CI) 1.9–46.4; $P = 0.006$], especially when the homozygous form was present (OR 44.2; 95% CI 5.5–354.6; $P < 0.001$). The A allele was less frequent in the high dose group showing an inverse association with the requirement for high doses (OR 0.04; 95% CI 0.01–0.22; $P < 0.001$). The A allele of the *c.*-1639G > A polymorphism of *VKORC1* is therefore associated with a low-dose requirement for acenocoumarol in patients receiving anticoagulant therapy.

Keywords: vitamin K, drugs, anticoagulation.

The coumarin derivative acenocoumarol is widely used to prevent recurrent arterial and venous thrombosis in European and Latin-American countries. The appropriate dose varies greatly and it is difficult to predict on an individual basis. Although this may partly be due to environmental factors, i.e. simultaneous exposure to other drugs that interfere with the metabolism of acenocoumarol, dietary habits or other health conditions, the inter-individual variability is thought to be largely determined by hereditary factors (Meyer, 2000). Among the genetic determinants, the variants *c.*430C > T (Arg144Cys) and *c.*1075A > C (Ile359Leu) of *cytochrome P450 2C9 (CYP2C9)*, involved in the metabolism of warfarin and acenocoumarol, have been shown to influence the anticoagulant dosage (Aithal *et al*, 1999; Daly & King, 2005). Progress has recently been made in our knowledge of the vitamin K cycle: the gene coding for the enzyme responsible for the transformation of vitamin K 2,3-epoxide to vitamin

K-hydroquinone has been cloned (Li *et al*, 2004; Rost *et al*, 2004). Vitamin K-hydroquinone is the co-factor for the carboxylase that adds a molecule of carbon dioxide to glutamic acid, producing γ -carboxyglutamic acid (γ -Glu). The γ -Glu residues are essential for some clotting factors, such as factors II, VII, IX, X and protein C, S and Z, to bind phospholipids and thus be functional. The enzyme, called vitamin K-epoxide reductase (*VKORC1*), is the target of the coumarin derivatives. Importantly, several genetic variations of the *VKORC1* gene have been found to influence sensitivity to warfarin (Rost *et al*, 2004; D'Andrea *et al*, 2005; Geisen *et al*, 2005; Quteineh *et al*, 2005; Rieder *et al*, 2005). Twenty-eight polymorphisms have been described in this gene, three main haplotypes covering almost the whole genetic variability of *VKORC1*. *VKORC1**2 haplotype has been found to account for most of the variability in response to oral anticoagulants (Geisen *et al*, 2005; Rieder *et al*, 2005; Sconce *et al*, 2005; Yuan *et al*, 2005). The

c.-1639G > A polymorphism marks the *VKORC1**2 haplotype (Geisen *et al*, 2005), and its association with increased response to acenocoumarol has been shown in a group of healthy subjects (Bodin *et al*, 2005). However, its influence in patients receiving acenocoumarol treatment have not been studied to date. Our aim was to study the effect of the *c.-1639G > A* polymorphism on the acenocoumarol dosage requirements in a group of patients under stable anticoagulation.

Materials and methods

Participants

A total of 113 outpatients attending the Hospital Clínico Universitario de Salamanca (Spain) between January and October 2001 who had maintained stable anticoagulation [International normalised ratio (INR) between 2 and 3·2] with acenocoumarol for at least 3 months were included in the study. We considered a patient on stable anticoagulation as one whose acenocoumarol dose requirement had remained constant for at least the previous 3 months, and this dose was the same (variation <25%) as the mean annual dose, and was enough to keep the INR within therapeutic ranges. Two patients with liver disease and two patients with thyroid disease were excluded. Four patients could not be analysed. The final population consisted of 42 consecutive patients requiring a low acenocoumarol dose (7 mg/week or less), 42 consecutive patients requiring a medium dose (>7 mg and <28 mg per week) and 21 consecutive patients requiring a high dose (28 mg/week or more). Low, medium and high weekly dose categories were arbitrarily chosen, taking into account the fact that around 80% of subjects in our population of patients under anticoagulation need a dose within the range defined as the medium dose (Hermida *et al*, 2002).

Data concerning age, sex, weekly dose required, exposure to other drugs affecting the acenocoumarol metabolism and the genotype of the *c.430C > T* and *c.1075A > C* variants of *CYP2C9* were recorded as described elsewhere (Hermida *et al*, 2002). All subjects had given informed consent to participate in the study.

VKORC1:c.-1639G > A genotyping

Genotyping was done by polymerase chain reaction followed by digestion with *Msp1* (New England Biolabs, Ipswich, MA, USA) according to described methods with minor modifications (Sconce *et al*, 2005). The forward and reverse primers were 5'-GCCAGCAGGAGAGGGAAATA-3' and 5'-AG-TTTGGACTACAGGTGCCT-3' respectively.

Statistical analysis

In order to evaluate the association between acenocoumarol requirements and the presence of the *A* allele at the -1639

position in the *VKORC1* gene, multivariable logistic regression models were fitted after adjusting for age, gender and the presence of other polymorphisms (*CYP2C9:c.430C > T* and *c.1075A > C*). We fitted two separate models. In both cases, the reference category was the group requiring a medium dose of acenocoumarol. In the first model, we estimated the odds ratio (OR) for requiring a low dose (excluding those with a requirement for a high dose). In the second model, we estimated the OR for requiring a 'high' dose (excluding those with a requirement for a low dose).

In the case of the *CYP2C9:c.430C > T* and *c.1075A > C* polymorphisms, heterozygous and homozygous subjects for the allelic variants were merged in the same category due to the low prevalence of homozygous subjects.

A linear regression model was also performed to assess the effect of the *A* allele of *VKORC1:c.-1639G > A* on the weekly dose of acenocoumarol (as a continuous variable) within the low dose group.

Interaction (effect modification) was assessed by introducing product-terms in the logistic regression models and considering any *P*-value < 0·05 for the likelihood ratio test of this product-term as evidence of a significant interaction.

In order to assess the proportion of carriers of both *VKORC1:c.-1639G > A* and *c.1075A > C* polymorphisms requiring a low acenocoumarol dose, we used exact methods [exact binomial confidence intervals (CI) and exact Fisher test]. Statistical analyses were performed with *STATA v9* software (StataCorp LP, College Station, TX, USA).

Results

Effect of VKORC1:c.-1639G > A on acenocoumarol dosage requirement

The distribution of the *G* and *A* alleles of *c.-1639G > A* polymorphism is shown in Table I. The allelic distribution was different across the various acenocoumarol dosage groups. Remarkably, in the group of 42 patients who maintained stable anticoagulation with a low dose of acenocoumarol, 38 patients

Table I. Frequency of the different genotypes for the *c.-1639G > A* polymorphism of the *VKORC1* gene in three groups of patients with different acenocoumarol requirements.

	Acenocoumarol dose requirement		
	Low dose (<i>n</i> = 42)	Medium dose (<i>n</i> = 42)	High dose (<i>n</i> = 21)
<i>GG</i>	4 (9·5%)	10 (23·8%)	15 (71·4%)
<i>GA</i>	12 (28·6%)	24 (57·1%)	6 (28·6%)
<i>AA</i>	26 (61·9%)	8 (19·1%)	0 (0%)

The number of carriers of each *VKORC1:c.-1639G > A* genotype, and the percentage (parentheses) of each genotype for each acenocoumarol dose are represented.

P for trend, < 0·001.

(90.5%) carried the *A* allele. However, the *A* allele was less frequent in the group of 21 patients requiring a high acenocoumarol dose: among these patients only 6 (28.6%) carried the *A* allele in the heterozygous form and none of them carried the *AA* genotype.

The mean age and the *c.430C > T* and *c.1075A > C* variants of *CYP2C9* in the three groups of patients have been reported previously (Hermida *et al*, 2002). We showed that both age and the polymorphisms of *CYP2C9*, especially the *c.1075A > C* variant, influenced the acenocoumarol dose requirements, while gender and other drugs did not (Hermida *et al*, 2002). We fitted logistic regression models to assess the independent contribution of *VKORC1:c.-1639G > A* to the inter-individual variability of the pharmacological response to acenocoumarol, taking also into account the effect of polymorphisms of *CYP2C9*, of age and sex. The results of this analysis are shown in Table II. The presence of the *A* allele increased the odds of requiring a low acenocoumarol dose. This effect was notably higher when the *A* allele was present in the homozygous form: OR 44.2 (95% CI 5.5–354.6; $P < 0.001$). Moreover, an inverse correlation was seen between the mean weekly dose of acenocoumarol required and bearing at least one *VKORC1:c.-1639A* allele (β coefficient, -0.35 ; $P = 0.043$) within the low dose group. Accordingly, not only did the *A* allele influence the odds of requiring a low acenocoumarol dose, but it also exerted an independent effect against requiring a high acenocoumarol dose.

The sensitivity and specificity of carrying the *VKORC1:c.-1639A* allele for low dose requirements were 90.5% (95% CI 77.4–97.3) and 40% (95% CI 27.6–52.8) respectively.

Effect of age and the variants of *CYP2C9*

The effect of age and the *c.430C > T* and *c.1075A > C* variants of *CYP2C9* on the acenocoumarol requirement in this

Table II. Association of the *VKORC1:c.-1639G > A* polymorphism, the *c.430C > T* and *c.1075A > C* variants of *CYP2C9* and age with the likelihood of requiring a low (A) or a high (B) dose of acenocoumarol.

	Crude OR	95% CI	Adjusted OR*	95% CI	<i>P</i> -value
A: low dose					
<i>VKORC1:c.-1639A</i>	2.96	0.85–10.37	9.38	1.90–46.43	0.006
<i>CYP2C9</i> variants	2.28	0.95–5.50	3.79	1.24–11.60	0.020
Age (years)	1.12	1.05–1.20	1.16	1.07–1.25	<0.001
B: high dose					
<i>VKORC1:c.-1639A</i>	0.12	0.04–0.41	0.04	0.01–0.22	<0.001
<i>CYP2C9</i> variants	0.21	0.04–1.07	0.06	0.01–0.62	0.017
Age (years)	0.94	0.89–0.99	0.90	0.84–0.97	0.005

OR, odds ratio; CI, confidence interval.

*Logistic regression models adjusted for gender and for the other variables shown in the table. The group of patients taking the medium dose of acenocoumarol was considered the reference category in both models.

population has been analysed and the results are available elsewhere (Hermida *et al*, 2002). However, at the time that work was carried out, the polymorphisms of the *VKORC1* gene had not yet been identified. For this reason, we updated the estimates for the independent association between both age and the variants of *CYP2C9* and the odds of requiring a low acenocoumarol dose after also adjusting for the *VKORC1:c.-1639G > A* polymorphism in the multivariate analysis. As can be observed in Table II, age was still an independent factor influencing the acenocoumarol dose after adjusting for the *c.-1639G > A* polymorphism: every additional year of age was associated with 16% higher odds of needing a lower acenocoumarol dose. Bearing the *c.430C > T* or *c.1075A > C* *CYP2C9* variants also remained independently associated with a higher odds of requiring a low acenocoumarol dose.

Combined effect of the *A* allele of *VKORC1:c.-1639G > A* and the *CYP2C9* variants

Remarkably, within the group requiring low acenocoumarol doses, all patients ($n = 42$) were carriers of at least one of the three polymorphisms analysed. In contrast, among the 21 patients who required a high acenocoumarol dose, 13 of them (61.9%) were free of the three polymorphisms. In the medium dose group, 36 of 42 subjects carried at least one of the three polymorphisms. All patients who were simultaneously carriers of both the *VKORC1:c.-1639G > A* allele and the *CYP2C9:c.1075A > C* polymorphism ($n = 10$) required a low acenocoumarol dose, whereas 11 of 20 patients (55%) carrying the *A* allele of *VKORC* and *CYP2C9:c.430C > T* belonged to the low acenocoumarol dose group. When an exact method (binomial CI) was used to assess the proportion of carriers of both *VKORC1:c.-1639A* and *CYP2C9:c.1075A > C* polymorphisms requiring a low acenocoumarol dose, the 95% CI estimated that 69–100% of patients that carried both mutations would require a low acenocoumarol dose ($P < 0.001$, Fisher exact test).

Discussion

The recent identification of the *VKORC1* gene (Li *et al*, 2004; Rost *et al*, 2004), involved in the vitamin K cycle, has prompted the search for new polymorphisms that can contribute to explaining the high interindividual variability in the dose requirements for oral anticoagulants. Much of this variability has been found to be due to the *VKORC1* genotype. Notably, 37% of the total variability in response to acenocoumarol in a group of healthy subjects was reported to be attributable to *VKORC1* (Bodin *et al*, 2005). Although several haplotypes have been identified in the *VKORC1* gene, those including the *c.-1639G > A* polymorphism have been associated with variability in the sensitivity to warfarin or acenocoumarol (Bodin *et al*, 2005; D'Andrea *et al*, 2005; Geisen *et al*, 2005; Rieder *et al*, 2005). Our findings support the importance of the role

played by the genotype *c.-1639G > A* in the sensitivity to acenocoumarol. The present study found that carrying the *A* allele notably increased the odds of requiring a low acenocoumarol dose to reach an INR within the therapeutic range. More than 90% of the patients who required a low dose of acenocoumarol to maintain a therapeutic INR carried the *A* allele, while this allelic variant was only present in <30% of the patients who needed a high dose. This allele was present in 76% of the patients in the medium dose group. Our findings support the notion that the association of *VKORC1:c.-1639G > A* with the sensitivity to acenocoumarol is larger than that observed with the *CYP2C9* polymorphisms, as the probability of requiring a low acenocoumarol dose associated with carrying the *A* allele was almost three times higher than the probability associated with the *CYP2C9* variants.

Although the characteristics of the present study did not allow us to determine how the variability differs for each polymorphism, from our data it can be inferred that genotyping *VKORC1:c.-1639G > A* and *CYP2C9:c.1075A > C* can help to identify a high percentage of the patients who can safely commence and maintain their anticoagulation on a low dose of acenocoumarol without risk of experiencing a bleeding episode. The 10 patients who simultaneously carried the *A* allele of *VKORC1:c.-1639A > G* and *CYP2C9:c.1075A > C* required a low dose of acenocoumarol for stable anticoagulation. Accordingly, among the 42 consecutive patients who entered the study because they required a low dose of acenocoumarol, 40 of them carried at least one of the two polymorphisms.

Our findings could be relevant when applied to clinical practice: as it has been suggested that the hyper-responsiveness to oral anticoagulant is linked to high risk of bleeding at the beginning of treatment, patients to be treated with acenocoumarol and bearing the *c.-1639G > A* polymorphism of *VKORC1* should start treatment with a reduced initial 'loading' dose in order to avoid over-anticoagulation and subsequent bleeding risk, particularly if they also carry the *c.1075A > C* variant of *CYP2C9*. An algorithm to calculate the daily dose of warfarin taking into account the *VKORC1* genotype has recently been proposed (Sconce *et al*, 2005). The present study could not build an algorithm because our population was not randomly selected.

The higher sensitivity to coumarin derivatives in the presence of the *A* allele of the *VKORC1:c.-1639G > A* polymorphism is probably due to a lower amount of *VKORC1* in these patients, which would lead to a decrease in the availability of vitamin K-hydroquinone: recently, an association between low hepatic *VKORC1* mRNA levels and haplotypes of the *VKORC1* gene carrying the *-1639A* allele has been shown, such patients being particularly sensitive to warfarin (Rieder *et al*, 2005).

This study has limitations: the sample size, although large enough to draw conclusions, was not sufficient to avoid wide CI; on the other hand, the patients were not randomly selected but chosen according to predefined groups (i.e. low, medium

and high acenocoumarol dose requirements). Hence, our results cannot be directly applied to the general population of patients receiving anticoagulants. Nonetheless, the strength of the association between the *VKORC1:c.-1639G > A* polymorphism and the acenocoumarol requirements undoubtedly underlines the importance of this genetic variant as a major determinant of the sensitivity to acenocoumarol in patients under anticoagulant treatment.

In conclusion, for the first time the association between the *c.-1639G > A* polymorphism of the *VKORC1* gene and the acenocoumarol requirement for stable long-term anticoagulation has been studied. The *A* allele increases the likelihood of requiring a low dose of acenocoumarol, especially when the *c.1075A > C* variant of the *CYP2C9* gene is also present. Genotyping for both mutations should make it possible to identify the vast majority of patients that are likely to require a low dosage of acenocoumarol.

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