Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity

Running title: pharmacogenetics of oral anticoagulant

Laurent Bodin¹, PharmD, Céline Verstuyft²³, PhD, PharmD, David-Alexandre Tregouet⁴, PhD, Annie Robert⁵, MD, Liliane Dubert³, BSc, Christian Funck-Brentano⁶, MD, PhD, Patrice Jaillon²⁶, MD, Philippe Beaune¹⁷, PhD, PharmD, Pierre Laurent-Puig¹⁷, MD, PhD, Laurent Becquemont²³, MD, PhD, Marie-Anne Loriot¹⁷, PhD, PharmD.

1 INSERM, UMRS 490; University René Descartes, Paris, F-75270 France.
2 Assistance Publique Hôpitaux de Paris; Pharmacology Department, Bicêtre University Hospital, University Paris-Sud, Le Kremlin Bicêtre, France.
3 Assistance Publique Hôpitaux de Paris; Pharmacology Department, Saint Antoine University Hospital - University Pierre et Marie Curie Paris 6, Paris, France.
4 INSERM, U525, Faculté de Médecine, Hôpital Pitié-Salpêtrière, Paris, France.
5 Department of Immunology and Hematology, Saint-Antoine Hospital, 184 rue du Faubourg Saint Antoine 75012 Paris, France.
6 Clinical Investigation Center, Saint-Antoine Hospital, 75012 Paris, France
7 Assistance Publique Hôpitaux de Paris; Biochemistry Department, European Georges Pompidou Hospital, Paris, France.

Correspondence:
Pr. Philippe Beaune

INSERM UMR S-490

Toxicologie Moléculaire

Centre universitaire des Saints-Pères

Université Paris5, René Descartes

45, rue des Saints-Pères

75270 Paris cedex 06

tel: +33(0)1 42 86 38 43

fax: +33(0)1 42 86 20 72

mail: Philippe.Beaune@univ-paris5.fr

Financial support: Supported by a grant from the Délégation Régionale de la Recherche Clinique d’Ile de France, Assistance Publique - Hôpitaux de Paris and by the Institut National de la Santé et de la Recherche médicale and the Assistance Publique –Hôpitaux de Paris, Clinical Investigation Centre, Saint-Antoine Hospital.

Dr Laurent Bodin received financial support from the French Ministère de la Recherche.

Key words: oral anticoagulant, VKORC1, CYP2C9, haplotype, acenocoumarol, pharmacogenetics, inter-individual variability.

Scientific heading: Hemostasis, Thrombosis, and Vascular Biology.

Abstract: 200 words

Full text: 3446 words
Summary

The aim of the study is to explore the contribution of genetic factors related either to drug metabolism (cytochrome P450 2C9) or to drug target (vitamin K epoxide reductase) to variability in the response to acenocoumarol among 222 healthy volunteers after a single oral dose. Associations between a pharmacodynamic index (reduction in Factor VII activity and INR change) and several genetic polymorphisms (VKORC1: -4931T>C, -4451C>A, -2659G>C, -1877A>G, -1639G>A, 497C>G, 1173C>T, and CYP2C9*3) were investigated using haplotype and univariate analyses. VKORC1 haplotypes were associated with the pharmacological response and this association can be explained only by the effect of the -1639G>A polymorphism (or alternatively by 1173C>T which is in complete association with it). Indeed, it explains about one third of the variability of the pharmacological response (37% of Factor VII decrease and 30% of INR change). Moreover, the previously observed effect of the CYP2C9*3 allele is independent of the VKORC1 gene effect. These two polymorphisms account for up to 50% of the inter-individual variability. The simple genotyping of two SNPs, VKORC1 –1639G>A or 1173C>T and the CYP2C9*3 polymorphisms, could thus predict a high risk of overdose before initiation of anticoagulation with acenocoumarol, and provide a safer and more individualized anticoagulant therapy.
INTRODUCTION

Oral anticoagulants, including warfarin and acenocoumarol (AC), are the most widely prescribed drugs for the prevention and treatment of arterial and venous thromboembolic disorders. However, warfarin was recently identified as a causal agent in 10% of cases of all hospital admissions for adverse drug reactions in England. The management of anticoagulant therapy is challenging because patients exhibit a large variability in their dose-anticoagulant effect response. A narrow range for a therapeutic effect makes individual dosing necessary. This inter-individual variability is known to depend on environmental factors but a genetic influence has also been demonstrated. The cytochrome P450 2C9 (CYP2C9) is the main enzyme responsible for the hepatic metabolism of oral anticoagulants such as acenocoumarol and warfarin. The CYP2C9 has been demonstrated to be polymorphic and its genetic variability has been shown to be associated with variations in the levels of enzyme activity.

Three major alleles have been found in humans: CYP2C9*1 (wild type or reference allele) Cys144/Leu359, CYP2C9*2, Arg144/Leu359 and CYP2C9*3, Cys144/Ile359. Due to an impaired metabolic capacity, patients having at least one CYP2C9*3 allele require, on average, a lower dose of AC than homozygotes for the CYP2C9*1 allele. Moreover, it has recently been shown in our laboratory that the CYP2C9*3 allele influences the AC response. Up to 14% of the inter-individual variability of the AC pharmacodynamic response can be explained based on this genotypic-haplotypic approach among caucasian subjects.

Recent works have also suggested that genetic variations within vitamin K-dependent protein genes could also be useful for predicting anticoagulant response. The vitamin K epoxide
reductase multiprotein complex 1 (\textit{VKORC1}) gene has been hypothesized to play a role in the variability of the AC response based upon a seminal study showing that an intronic 1173C>T polymorphism was associated with warfarin dose requirement. Patients carrying the T allele required a lower dose of warfarin as compared with those carrying the C allele (the mean daily dose decreased 43\% for homozygous TT carriers and 22\% for heterozygous).\textsuperscript{16} The aim of this study was to evaluate the contribution of genetic variability of the \textit{VKORC1} gene, in addition to the 1173 C>T polymorphism, on the pharmacodynamic outcome after AC intake.
METHODS

Subjects

The study population was the same as the one used in our previous analysis of the CYP2C9 haplotype. Two hundred and sixty-three healthy volunteers between 18 and 65 years old were enrolled in this study. Among them, 230 were of caucasian origin and DNA samples were available for 222 of them (145 males and 77 females). Each subject provided written informed consent before participation. The study was approved by the «Comité consultatif pour la protection des personnes participant à la recherche biomédicale» of Pitié-Salpêtrière Hospital (local ethical committee) and complies with French bioethics laws. This study took place at the Center for Clinical investigation of Saint-Antoine University Hospital, Paris.

The CYP2C9 genotype was previously determined. The observed frequencies of minor alleles CYP2C9*2 and CYP2C9*3 among the 222 subjects studied in the present setting were respectively of 0.14 and 0.086.

Methods

Selection of polymorphisms in VKORC1 gene

The VKORC1 gene polymorphisms studied in this report were selected from public databases (http://pga.gs.washington.edu/data/vkorc1/welcome.html). Twenty-nine polymorphisms have been identified from a population including 24 African individuals and 23 European individuals and thirteen haplotypes have been inferred (frequency ranged from 1 to 17%) from 17 SNPs with allele frequency greater than 4% (http://pga.gs.washington.edu/data/vkorc1/welcome.html). All the polymorphisms described
by D’Andrea et al with a frequency greater than 1% were listed within this database (i.e. 3462 C>T in exon 3, 1173 C>T in intron 1, 3730 G>A in the 3’UTR) and among the 17 above-mentioned SNPs.

For the present study, we used the TAG’n’TELL software (http://snp.cgb.ki.se/tagntell/) to identify and select the minimum number of SNPs that capture more than 95% of the haplotypic diversity referred as the "haplotype tagging SNPs" (htSNPs). 17 Six SNPs were selected in this way: five (-4931 T>C, -4451 C>A, -2659 G>C, -1877 A>G, -1639 G>A) located in the 5’flanking region of the VKORC1 gene and one (497 C>G) in intron 1 (table 1).

In view of the recent findings suggesting a potential effect of the 1173 C>T intronic polymorphism on the pharmacodynamic response, this SNP was also selected for genotyping even though it was not identified as a htSNP.

Genotyping of VKORC1 htSNPs (-4931 T>C, -4451 C>A, -2659 G>C, -1877 A>G, -1639 G>A, 497 C>G) and 1173 C>T SNP

For the –4931 T>C polymorphism, a real-time PCR allelic discrimination assay was designed using Primer Express® software and employed a forward primer (5’GCAACATAACAACAACACTCATCTCTAATG 3’), a reverse primer (5’GGCCAGGCTGGTCTCAAAC 3’) and two MGB probes namely 5’Fam-ATCCaCCCTCCTTGG 3’ (as the wild type genotype) and 5’Vic-ATGATCCgCCCTCC 3’ (as the mutated genotype). Amplification was performed using a 7900HT® Applied Biosystems real-time thermal cycler. All probe/primer sets were designed to function using universal reaction and cycling conditions.
Direct sequencing after PCR was performed for the detection of all other SNPs. The primer sequences are available on request. Sequencing was performed on an ABI Prism Genetic Analyzer System 9700 (Applied Biosystems, Courtabœuf, France).

**Pharmacodynamics**

INR was determined using Thromborel S (Dade Behring, Marburg, Germany). Factor VII coagulant activity was determined by a one stage assay using factor VII-deficient plasma (Dade Behring) and Thromborel S.

To evaluate AC pharmacodynamics, Factor VII ratio ([Factor VII 24h after AC intake / Factor VII before AC intake]x 100) and INR change (i.e.[(INR 24h after AC intake- INR before AC intake) / INR before AC intake] x100) were chosen to evaluate AC pharmacodynamics.

**Plasmid construction**

To study the effect on the promoter activity of the VKORC1 –1639 G>A polymorphism, a 2171 bp fragment containing either the G (wild type) or A (mutant) allele was initially amplified from genomic DNA (isolated from subjects homozygous either for the G or A allele) with VKORC1 specific primers (5'-AGGAGATTGGTCAGCTTAATTCCAT-'3 and 5'-TAATCATCTGGCATCCTGGC-3'). Finally, a 1768 bp subfragment (-1756, -12; the nucleotides are numbered from the A in the ATG initiating translation) was obtained from the first PCR product with primers incorporating NheI and HindIII restriction sites (5'-GGAGGAGCTAGCAGGAGAGGG-3’ and 5’-TATCTCAAGCTTCCGCCCCG-3’).

The two purified PCR products (e.g. one containing the G allele, the other the A allele) were then digested with HindIII and NheI and cloned in the pGL3 basic vector, upstream of the
Firefly luciferase reporter gene (Promega, Charbonière, France). The PCR reactions were run with proofreading Pfu™Turbo DNA polymerase (Stratagene, Charbonière, France). All constructs were checked by direct sequencing on an ABI PRISM 7700 Genetic Analyser sequencer (Applied Biosystems, Courtaboeuf, France). The \( \beta \text{glob-RL} \) plasmid, a gift of Dr Y. Morel, contains a promoter derived from the proximal part of the human \( \alpha \)-globin promoter upstream of the Renillla luciferase reporter gene (used as internal control) \(^{18} \) which allows control of the transfection efficiency.

**Cell culture and transfection**

HEPG2 cells were grown in 50% DMEM (Dulbecco's modified Eagle's medium) and 50% HamF12 medium supplemented with 10% FBS (fetal bovine serum), 200 U/mL penicillin, 50 \( \mu \text{g/mL} \) and 0.5 mg/mL fungizone in a humidified atmosphere in 5%. Transfection experiments were performed in HepG2 cells using the calcium phosphate method. Briefly, the day prior the transfection, cells were trypsinised and seeded (2 \( \times \) 10\(^5 \) in 12-well plates) into the usual culture medium. For each series of transfections, 1\( \mu \text{g} \) of pGL3 basic plasmid containing either the \( \text{VKORC1} \) wild type or mutated promoter construct, 100 ng of \( \beta \text{glob-RL} \) plasmid were added to 31 \( \mu \text{l} \) of 2M CaCl\(_2 \) and mixed with an equal volume of 2x HBS (HEPES Buffered Saline) buffer. After a 20 min incubation, 500 \( \mu \text{l} \) of this mixture was added to each well containing 2 ml of media. The media was changed 4 h post transfection. The luciferase activity was measured by using the Dual luciferase (Firefly and Renilla) assay kit (Promega, Charbonière, France) 48 hours later according to the manufacturer ‘s instructions.
Results of wild type and mutant constructs were analyzed by expressing the ratio of the two luciferase activities. Renilla luciferase activity was used to normalize the transfection efficiency in all culture dishes. Each assay was repeated in four independent experiments including 6 replicates for the two constructs.

**Statistics**

One-way ANOVA with Dunnett's post test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Allele frequencies were estimated by gene counting. Departure from Hardy-Weinberg equilibrium was tested by a Chi² test with 1 df. ANOVA analysis was first performed to test for the association of each *VKORC1* polymorphisms with AC response and then followed by a haplotype-based association analysis to take into account the simultaneous information of all *VKORC1* polymorphisms.

Linkage disequilibrium (LD) and haplotype analyses were performed by use of the THESIAS software (http://ecgene.net/genecanvas/modules/mydownloads/singlefile.php?cid=1&lid=4) based on the SEM algorithm. This method allows the estimation of haplotype frequencies and phenotypic means according to the carried haplotypes under the assumption of additive haplotypic effects. Phenotypic changes, expressed as a percentage compared to baseline values, before AC intake, was reported with its corresponding 95% confidence interval. Finally, in order to test whether the *CYP2C9*^*3* allele previously shown to be associated with Factor VII variability could interact or act additively with *VKORCI* SNPs, interaction was
investigated though multiple regression analysis by introducing genotype x genotype interaction terms in the regression model.
RESULTS

Allele frequencies of VKORC1 gene among 222 healthy volunteers

Allele frequencies of the seven SNPs studied are reported in table 1. No significant deviation from Hardy-Weinberg equilibrium was observed for any polymorphism (p>0.05). The observed frequencies of minor alleles ranged from 0.21 to 0.42, except for the -1877 A>G that appears with a lower frequency (0.02).

Effect of VKORC1 genotypes on AC pharmacodynamic response

All polymorphisms studied were strongly associated with the Factor VII ratio and INR change (p< 10^-4). The Factor VII ratio phenotypic variance explained by genotype ranged from 8% for the –2659 G>C polymorphism to 37% for the –1639 G>A polymorphism. Corresponding values for INR change ranged from 7% to 30% for the same polymorphisms. The Factor VII ratio (fig 1A) and the INR change (%) (fig 1B) are plotted against VKORC1 genotype (only the –1639 G>A SNP, that corresponds to the more informative SNP, is shown, see below). The Factor VII ratio after AC intake decreased in the following order: VKORC1 -1639 G>A genotype (70% ± 14 for VKORC1 –1639 G/G, 56% ± 15 for VKORC1 1639 G/A and 35% ± 17 for VKORC1 –1639 A/A, p<0.01). The same effect was observed for INR change, with an increase of INR more marked in subjects carrying the VKORC1 –1639 A allele (12% ± 9 for VKORC1 –1639 G/G, 21% ± 14 for VKORC1 –1639 G/A and 42% ± 22 for VKORC1 –1639 A/A, p<0.01).

VKORC1 haplotype analysis
All polymorphisms were in strong linkage disequilibrium (LD) with each other. In particular, the 1173 C>T and -1639 G>A polymorphisms were in complete disequilibrium. As a consequence of this LD pattern, 7 haplotypes were inferred, among which 5 appeared with a frequency greater than 0.05 and accounted for about 95% of all chromosomes (Table 2). The rare -1877 G allele seemed to be carried out by several haplotypes with very low frequency (0.007) and is therefore not depicted in Table 2. VKORC1 gene haplotypes were highly associated with Factor VII ratio ($\chi^2 = 95.43$ with 6 df, $p < 10^{-4}$) and explained 37% of its variability. Interestingly, all three haplotypes carrying the -1639A (or the 1173T) allele were associated with low Factor VII ratio levels (~19%) or higher INR change (~19%) while all those carrying the -1639G (or the 1173C) allele were associated with increased levels of Factor VII ratio (~36%) or lower INR change (~5%) suggesting that the data could be compatible with the sole effect of the –1639 G>A (or 1173 C>T) polymorphism. This hypothesis was not rejected ($\chi^2 = 2.40$ with 5 df, $p = 0.79$). Thus, in subjects with the –1639 A, AC has a significantly higher pharmacological effect than in subjects with the –1639 G.

The same analysis, including INR change as quantitative variable, led to results similar to those obtained with Factor VII ratio (e.g. 30% of variability for VKORC1).

Multiple linear regression model

*CYP2C9*3 allele was demonstrated in a previous paper \(^{12}\) to account for 14% of variability in percentage of remaining Factor VII. Multiple regression analysis revealed that the *CYP2C9*3 and the -1639 G>A polymorphisms were independently associated with AC response and
explained up to 50% of the variability in the AC response. When the subject’s body weight was combined with \textit{CYP2C9} and \textit{VKORC1} SNPs, an additive effect was observed, increasing the part of variability explained by these 3 parameters to 54%.

Results similar to those obtained with Factor VII ratio were observed when using INR change as the pharmacological index (e.g. 40% of variability when combining subject’s weight with \textit{CYP2C9} and \textit{VKORC1} SNPs).

In table 3, the combined effects of the \textit{CYP2C9} and \textit{VKORC1} genotypes are presented and plotted against the Factor VII ratio (table 3A) and INR change (table 3B). The additive effect of the \textit{VKORC1} -1639 G>A SNP and the \textit{CYP2C9*3} allele is observed, when comparing pharmacological response between subjects carrying the \textit{CYP2C9*3} and/or the \textit{CYP2C9*1} allele within the \textit{VKORC1} genotype, and inversely.

\textbf{Luciferase reporter gene assays}

We have cloned a 1.7-kb fragment of the 5’ flanking region of the \textit{VKORC1} gene containing either the −1639 G or −1639 A allele and placed it upstream of the luciferase gene in the pGL3-Basic vector in order to analyze its transcriptional activity in HepG2. For normalization of luciferase activity, the pglob-RL plasmid containing a promoter encoding Renilla luciferase, was used. The pglob-RL plasmid was used together with each pGL3 plasmids for co-transfection. Two co-transfections were performed containing either the −1639 G and the pglob-RL plasmid or −1639 A allele and the pglob-RL plasmid. The level of firefly luciferase activity was normalized to that of the Renilla luciferase activity in each experiment and the activity of each promoter construct was similar to the other promoter construct used in
our laboratory. The percentage of luciferase activity after normalization was similar for the two plasmid constructs (data not shown).
DISCUSSION

The aim of the present study was to investigate the part of CYP2C9 and VKORC1 genetic polymorphisms on the inter-individual variability of the AC pharmacodynamic response. The design study included healthy caucasian volunteers thus allowing the determination of the genetic component of the pharmacological response while permitting adequate control of non genetic parameters (foods, drugs, pathological situations) known to influence the response. The results of this study demonstrate that genetic polymorphisms in CYP2C9 and VKORC1 account for about 50% of the variability in the AC pharmacological response. A recent publication by D’Andrea et al. suggested, for the first time, the role of VKORC1 genetic polymorphisms on warfarin dose requirements. Our data confirm the impact of VKORC1 genetic variations, taking into account all of the known frequent polymorphisms listed within the gene (5’UTR, coding region and the 3’UTR) and using haplotype analysis.

The dose of AC is currently adjusted according to the INR but a large variability in the dose-response relationship exists which explains the frequent complications: bleeding or insufficient anticoagulation. As described elsewhere, these variations in dose-response may be due partly to differences in pharmacokinetics and genetic polymorphisms in CYP2C9, as shown by the results of this and other studies, are not the only factors involved in inter-individual differences. Indeed, AC metabolism is influenced by a number of pathologic, physiologic, and environmental factors. In a previous study, we have shown that body weight and one CYP2C9 haplotype accounted for about 19% of the variability in the anticoagulant effect of AC. To improve and extend these findings, the VKORC1 gene was
also studied. Our results show that the VKORC1 genotype explains 37% of the variability, demonstrating the major role of this gene in the pharmacological response. According to the design of our study, the percentage of remaining Factor VII (or Factor VII ratio) was the most sensitive parameter for the measurement of the AC pharmacological response. However, similar results were also observed when using INR change as a phenotypic variable. It is usually determined 48 or 72 h after anticoagulant drug intake because the half lives of other blood coagulation factors (e.g. Factor II, IX and X) are greater than 48 h (up to 60 h for the Factor II). Since the pharmacological response was calculated over 24h, the association between VKORC1 genotype and the INR change might have been underestimated.

This is the first study that reports the effect of genetic polymorphisms of the VKORC1 gene by haplotypic analysis in the variability to the AC response. The htSNPs were identified from data obtained from sequencing of a panel of 47 selected individuals (http://pga.gs.washington.edu/data/vkorc1/welcome.html) in order to perform haplotype construction and then, to determine the haplotypic effect on the pharmacodynamic response to AC. As shown in table 1, the frequencies observed in our population (222 subjects) are similar to those observed in the panel of 47 samples for all SNPs, except for the -1877A>G mutation (0.02 versus 0.14). This difference could be explained by the genetic heterogeneity between the ethnic groups. Further, the association of each SNP individually with the percentage of remaining Factor VII and INR change was tested. These SNPs are significantly correlated with the AC response and allowed the identification of seven major VKORC1 haplotypes (table 2). Haplotypic analysis revealed that the –1639 G>A polymorphism (or the
1173 C>T polymorphism in complete association with it) is sufficient to explain all of the pharmacological variability (~37%). It is noteworthy that the htSNPs are currently used as genetic markers (for haplotypic information) independently of the functional impact. In this case, the use of MatInspector (Genomatix Software GmbH, Munich, Germany) has revealed a potential nuclear factor 1 (NF1) binding site motif (TTGGCCA) in the VKORC1 gene at position -1639 upstream of ATG initiation codon. NF1 is an ubiquitous transcription factor known to play an important role in viral and cellular regulation and is involved in the control of constitutive and inducible gene expression. Thus, a possible explanation would be that the mutation -1639G>A modifies a putative binding site for NF1 affecting VKORC1 transcription. To test this hypothesis, a luciferase reporter gene assay was performed. Our results showed no difference between the wild type and the mutant promoter. D’Andreas et al found that, the 1173C>T intronic mutation, associated with low dose requirement of warfarin, did not affect the processing of VKORC1 mRNA. Altogether, these results suggested that a clear functional SNP in VKORC1 gene was not yet identified, although both SNPs were linked in the pharmacological response to anticoagulant.

The multiple linear regression analysis indicated that the genetic polymorphisms of CYP2C9 and VKORC1 strongly modulate the pharmacological response to AC with an additive effect, and, based upon our previously published data, body weight, CYP2C9 and VKORC1 accounted for about 54% for the variability. The results concerning these factors will likely have implications not only for AC but also for other oral anticoagulant treatments such as warfarin and phenprocoumon which are metabolized by the same enzymes and have the
same targets (vitamin K dependent enzymes). Thus, the inclusion of these parameters in computer-generated algorithms to determine the initial doses of these drugs after a simple genotyping (the 2 SNPs described in this study for AC and the additional CYP2C9*2 for warfarin and phenprocoumon). Our results should be confirmed by prospective clinical studies including treated patient populations. The knowledge of the pharmacogenetics of drug metabolism and targets for vitamin K antagonists raises the possibility to individualize the initial dose and, thus, to limit the frequent complications observed with the use of these drugs.

In conclusion, the joint genotyping of two SNPs which is easy and inexpensive, CYP2C9*3 and -1639 G>A or 1173 C>T on the VKORC1 gene predicts about 50% of the inter-individual variability of the AC pharmacodynamic response providing the opportunity for safer anticoagulant therapy. For the sake of clarity, a consensus choice should be made between -1639 G>A and 1173 C>T as definitive Tag SNP. Pharmacogenetic testing could predict a high risk of overdose among 25% of population before initiation of anticoagulation. This would correspond to the subjects carrying either the VKORC1 variant allele (-1639A or 1173 T) or the CYP2C9*3 allele. Some authors have found a weak effect of CYP2C9*2 on the dose of acenocoumarol necessary to provide the expected anticoagulation. In this paper we did not found such difference as some other authors and as discussed in our previous paper. The improvement of coumarinic drug (acenocoumarol, warfarin) management is clinically relevant because of the wide use of these drugs and because of the difficulties encountered during the development of the novel anticoagulants.
Acknowledgements

We thank all the members of the Clinical Investigation Center of Saint-Antoine and of the INSERM U490 for their skillful technical assistance. We are grateful to Drs I. de Waziers, C. Tomkiewicz and A. Moncion for assistance with the cloning and cell culture work. The authors wish to thank Dr Lawrence P. Aggerbeck for help in the manuscript correction.
References


Table 1
Position and frequencies of \textit{VKORC1} genetic polymorphisms and SNPs used for haplotype construction.

† the position of the SNP is relative to the A of the ATG initiation codon (denoted nucleotide +1).

‡ designation of the SNP relative to the first nucleotide referred to as accession number AY587020 (NCBI) or in http://pga.gs.washington.edu/data/vkorc1/welcome.html. The length of the analyzed \textit{VKORC1} sequence (including 5’flanking, exonic, intronic and 3’ untranslated regions) is of 12 Mb, and the bases are numbered from 1 to 12000.

§ the indicated frequencies correspond to those of the minor allele.

<table>
<thead>
<tr>
<th>SNPs gene location</th>
<th>Frequency</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>† -4931 T&gt;C</td>
<td>381</td>
<td>0.47 0.42</td>
</tr>
<tr>
<td>† -4451 C&gt;A</td>
<td>861</td>
<td>0.26 0.33</td>
</tr>
<tr>
<td>† -2659 G&gt;C</td>
<td>2653</td>
<td>0.17 0.21</td>
</tr>
<tr>
<td>† -1877 A&gt;G</td>
<td>3435</td>
<td>0.14 0.02</td>
</tr>
<tr>
<td>† -1639 G&gt;A</td>
<td>3673</td>
<td>0.29 0.42</td>
</tr>
<tr>
<td>† 497 T&gt;G</td>
<td>5808</td>
<td>0.18 0.30</td>
</tr>
<tr>
<td>† 1173 C&gt;T</td>
<td>6484</td>
<td>0.31 0.42</td>
</tr>
<tr>
<td>VKORC1 SNPs</td>
<td>Phenotypic change</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td><strong>-4931 T&gt;C</strong></td>
<td><strong>Factor VII ratio (%) (95% CI)</strong></td>
<td><strong>INR change (%) (95% CI)</strong></td>
</tr>
<tr>
<td>C C G A G T</td>
<td>19 (17-21)</td>
<td>18 (16-20)</td>
</tr>
<tr>
<td>C C G A T T</td>
<td>19 (14-23)</td>
<td>19 (16-22)</td>
</tr>
<tr>
<td>T C G A G T</td>
<td>19 (8-31)</td>
<td>16 (4-28)</td>
</tr>
<tr>
<td>T C G G T C</td>
<td>34 (32-37)</td>
<td>7 (4-10)</td>
</tr>
<tr>
<td>T A C G T C</td>
<td>37 (34-40)</td>
<td>4 (-0.7-8)</td>
</tr>
<tr>
<td>T A G G T C</td>
<td>38 (33-42)</td>
<td>3 (-2-9)</td>
</tr>
<tr>
<td>C C G G T C</td>
<td>42 (22-61)</td>
<td>-2 (-18-14)</td>
</tr>
</tbody>
</table>

Table 2

Haplotypic analysis of the VKORC1 gene among the 222 healthy volunteers in relation to acenocoumarol response.

Construction and estimated frequencies of haplotypes were provided by new THESIAS (http://ecgene.net/genecanvas/modules/mydownloads/singlefile.php?cid=1&lid=4).
Phenotypic changes are expressed as the percentage, compared to baseline values, before AC intake, of Factor VII ratio or INR change resulting from the one-dose effect of haplotype (each individual carrying two haplotypes).

Factor VII ratio corresponds to remaining factor VII, expressed as a percentage compared to baseline value, before AC intake (e.g. [Factor VII 24h after AC intake / Factor VII before AC intake] x 100).

INR change is expressed as the formula: [(INR 24h after AC intake - INR before AC intake / INR before AC intake] x 100.

The low frequency of the SNP -1877A>G does not permit the study of the effect on AC response of the corresponding haplotype (frequency= 0.007).

Negative values for phenotypic change in the INR change column reflect the low variation among this haplotype.

Likelihood ratio test was used for a global haplotypic effect: $\chi^2 = 95.43$ with 6 df, $p < 10^{-4}$. 
Table 3

Combined effect of CYP2C9 and VKORC1 genotypes on the percentage of Factor VII ratio (3A) and INR change (3B) after a single dose AC intake.

The combined genotypes of VKORC1 (G/A and A/A) and CYP2C9 (CYP2C9*3 allele) represent 25% of the population.

Average of percentage of remaining Factor VII and INR change is represented within different CYP2C9 and VKORC1 genotypes.

The numbers of the VKORC1 G/G, G/A and A/A genotypes among the CYP2C9*1/*1 individuals were 59, 95, and 30, respectively.

The numbers of the VKORC1 G/G, G/A and A/A genotypes among the heterozygous carriers of CYP2C9*3 allele were 13, 15, and 10, respectively.
Figure 1
Legend of the figure

Figure 1

Factor VII ratio (1A) and INR change (1B) according to *VKORC1* genotype after a single oral-dose of acenocoumarol.

Factor VII ratio corresponds to remaining factor VII, expressed as a percentage compared to baseline value, before AC intake (e.g. \([\text{Factor VII 24h after AC intake} / \text{Factor VII before AC intake}] \times 100\)).

INR change is expressed as the formula: \([\left(\frac{\text{INR 24h after AC intake} - \text{INR before AC intake}}{\text{INR before AC intake}}\right) \times 100]\).

Bars represent means; Factor VII ratio and INR change were significantly different between each intra-group.

*** \(p<0.01\)
Abbreviations and symbol

SNP, single nucleotide polymorphism; AC, acenocoumarol; htSNP, haplotype tagging SNP;
INR, International Normalized Ratio
Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity

Laurent Bodin, Céline Verstuyft, David-Alexandre Tregouet, Annie Robert, Liliane Dubert, Christian Funck-Brentano, Patrice Jaillon, Philippe Beaune, Pierre Laurent-Puig, Laurent Becquemont and Marie-Anne Loriot