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ORIGINAL ARTICLE

Common *VKORC1* and *GGCX* polymorphisms associated with warfarin dose

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ABSTRACT

We report a novel combination of factors that explains almost 60% of variable response to warfarin. Warfarin is a widely used anticoagulant, which acts through interference with vitamin K epoxide reductase that is encoded by *VKORC1*. In the next step of the vitamin K cycle, gamma-glutamyl carboxylase encoded by *GGCX* uses reduced vitamin K to activate clotting factors. We genotyped 201 warfarin-treated patients for common polymorphisms in *VKORC1* and *GGCX*. All the five *VKORC1* single-nucleotide polymorphisms covary significantly with warfarin dose, and explain 29–30% of variance in dose. Thus, *VKORC1* has a larger impact than cytochrome *P*450 2C9, which explains 12% of variance in dose. In addition, one *GGCX* SNP showed a small but significant effect on warfarin dose. Incorrect dosage, especially during the initial phase of treatment, carries a high risk of either severe bleeding or failure to prevent thromboembolism. Genotype-based dose predictions may in future enable personalised drug treatment from the start of warfarin therapy.

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INTRODUCTION

Warfarin is the most widely prescribed anticoagulant for thromboembolic therapy in North America and Europe.[1] It is used in atrial fibrillation, recurrent stroke, deep vein thrombosis, pulmonary embolism, and in patients with heart valve prosthesis.[2,3] The therapeutic response is possible to measure by the prothrombin time international normalised ratio (PT INR);[4] nevertheless, warfarin is difficult to handle because of a narrow therapeutic range and a 20-fold inter-individual variation in dose requirement.[5,6] Incorrect dosage, especially during the initial phase of treatment, carries a high risk of either severe bleeding or failure to prevent thromboembolism.[6] Haemorrhage during warfarin therapy is actually one of the leading causes of drug-related death in many Western countries.[6–10]

Environmental and genetic factors influence the dose of warfarin necessary for a therapeutic response.[11] Factors such as age, bodyweight, diet and concomitant medication are well known to affect dose requirement.[3,12–14] Variation in warfarin pharmacokinetics is another important factor influencing dose.[15] Warfarin is administered as a racemate that consists of R- and S-enantiomers, the S-form being three to five times more active than the R form.[16,17] S-warfarin is metabolised by the cytochrome *P*450 enzyme CYP2C9,[18] and genetic variability in *CYP2C9* partly explains the large differences in the required

warfarin dose.[15] Two variants that encode enzymes with single amino-acid substitutions, *CYP2C9*2* and *CYP2C9*3*, are associated with a significant decrease in warfarin dose requirement, especially among Europeans.[19] A large number of studies concerning the influence of *CYP2C9* genotype on warfarin dose have been published.[1–3,5,11,20–24]

There is as yet limited information regarding pharmacodynamic factors involved in variable response to warfarin. Warfarin acts through interference with the vitamin K cycle in the liver (Figure 1).[25] It limits the regeneration of reduced vitamin K and, thus, limits the production of active clotting proteins.[26] The enzyme vitamin K epoxide reductase (VKOR) was identified as warfarin's target 30 years ago,[27,28] but the actual enzyme proved difficult to purify.[29] The gene of the major protein component of VKOR was eventually mapped to human chromosome 16p12–q21,[30] and it was recently identified as VKOR complex subunit 1 (*VKORC1*).[31,32] *VKORC1* spans 4 kilobases (kb), consists of three exons (NM_024006), and encodes a 163 amino-acid enzyme located in the endoplasmic reticulum (NP_076869). Rare mutations that lead to familial defective vitamin K-dependent clotting factors and hereditary warfarin resistance have been found in human *VKORC1*.[31,33]

Reduced vitamin K is a required cofactor for the activation of clotting factors II, VII, IX and X and proteins C, S and Z by gamma-glutamyl carboxylation (Figure 1).[34] Human gamma-glutamyl carboxylase (GGCX) was identified almost 30 years ago.[35] In the mid-90s, the human enzyme was purified,[36] and the gene (*GGCX*) was mapped to human chromosome 2p12.[37,38] *GGCX* spans 13 kb, contains 15 exons (NM_000821) and codes for a 758 amino-acid membrane protein of the endoplasmic reticulum and the Golgi apparatus (NP_000812).[35,39] Two rare mutations in *GGCX* that cause deficiency of all vitamin K-dependent coagulation factors have been identified.[34]

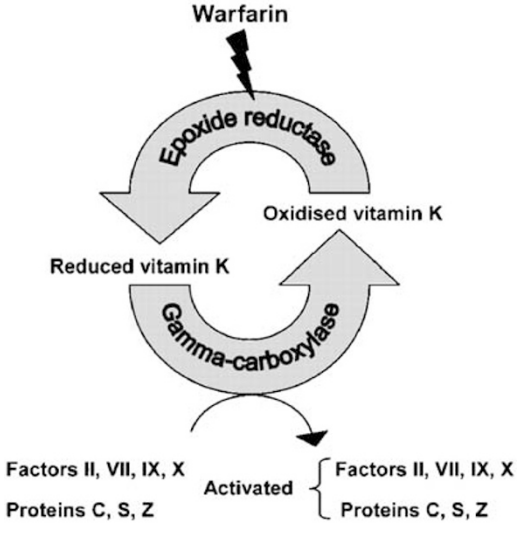

Figure 1 The vitamin K cycle.[25] VKOR reduces vitamin K. Reduced vitamin K is a cofactor for activation of clotting factors II, VII, IX and X and proteins C, S and Z by GGCX. In the process vitamin K is oxidised, and in the next cycle VKOR regenerates reduced vitamin K. Warfarin inhibits VKOR, impairing the synthesis of clotting factors.

We attempt to evaluate whether common genetic variation in the vitamin K cycle affects warfarin dose requirement in an unselected population at a hospital anticoagulation clinic. Here we report a novel combination of factors that allows, for the first time, explanation of almost 60% of variable response to warfarin.

RESULTS

We selected all publicly available (dbSNP 121) single-nucleotide polymorphisms (SNPs) in the *VKORC1* (chr16: 31013777–31009681 bp; NCBI build 35) and *GGCX* (chr2: 85700237–85687865 bp) genes, including 5 kb up- and downstream flanking regions. We designed MassExtend assays for 29 *VKORC1* SNPs, including the mutations reported by Rost *et al*,[40] and for 16 *GGCX* SNPs and used them to type 201 warfarin-treated patients. Only SNPs without significant deviation from Hardy–Weinberg equilibrium were used in the statistical evaluation of the study.

We obtained results for 20 *VKORC1* SNPs, with five of them being common in this sample. Four SNPs have minor allele frequencies (MAF) around 40% and are located 5′ upstream (rs9923231), in the first intron (rs9934438), second intron (rs2359612) and 3′untranslated region (UTR) (rs7294) of the gene (Figure 2). The fifth SNP (rs11150606) is located downstream of the gene and has a MAF of 4%. Inter SNP distances are 2.8, 1.1, 1.5 and 3.3 kb, respectively. We applied the confidence intervals method with the HaploView software.[40] We found that the four most common SNPs are in strong linkage disequilibrium (LD), and give rise to three common haplotypes that are further subdivided into four by SNP rs11150606 (Figure 2). Note that, based on HapMap data (http://www.hapmap.org), this region of high LD extends up to 285 kb in Caucasians.

For *GGCX*, we obtained results for 14 SNPs, and, as shown in Figure 3, nine SNPs have MAF above 30%. They are located in intron one (rs7568458), intron two (rs12714145), intron five (rs6738645), intron six (rs762684), exon eight (rs699664), exon nine (rs2592551), intron 14 (rs2028898) and in the 3′ flanking region (rs6547621 and rs7605975). The exon eight SNP rs699664 leads to an arginine to glutamine change in codon 325, while rs2592551 in exon nine is synonymous. Inter-SNP distances are 0.8, 4.2, 1.1, 1.5, 0.4, 2.9, 2.6 and 2.1 kb, respectively. All nine SNPs are within a region of strong LD and define five common haplotypes (Figure 3).

Alleles of the five *VKORC1* SNPs covary significantly with warfarin dose according to a regression model. Four of them are extremely closely associated with dose, $P<0.0001$ (Figure 4), while the fifth (rs11150606), which is located downstream of the gene and has a lower MAF, shows a less significant association, $P=0.0221$. All five SNPs are good predictors of warfarin maintenance dose, and explain 29–30% of inter-individual variability (Table 1). In the

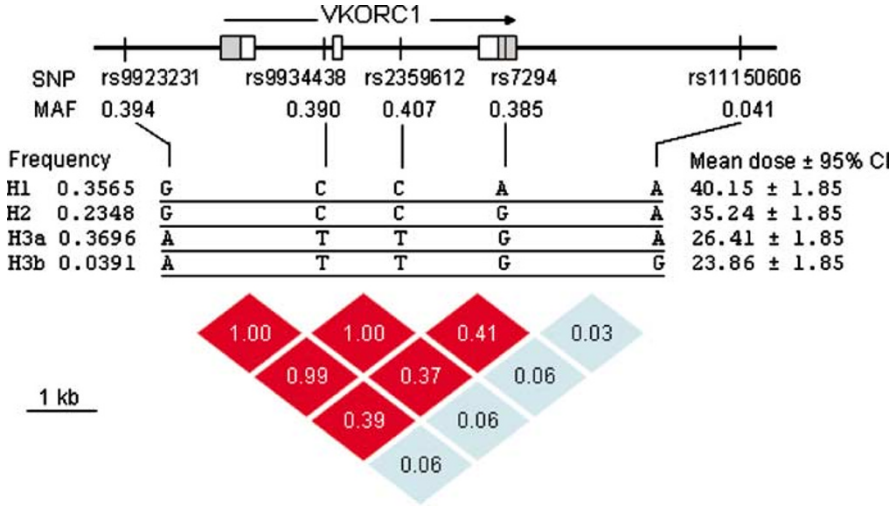

Figure 2 Genomic organisation, LD and common haplotypes across the *VKORC1* gene. Shaded and open rectangles indicate the UTR and coding parts of exons, respectively, with exon positions along chromosome 16 (left to right) being 31013777–31013379, 31012243–31012134 and 31010164–31009677 bp (NCBI 35). The position of the ATG codon is at 31013551 bp. rs9923231 is located 1639 bp upstream of the ATG codon at 31015190 bp, whereas rs9934438, rs2359612, rs7294 and rs11150606 are located downstream of the ATG codon at positions 1173 (31012379 bp), 2255 (31011297 bp), 3730 (31009822 bp) and 7040 (31006512 bp), respectively. The MAF of each SNP is given, with pairwise LD displayed in red (strong LD) and light blue (low LD) rectangles; values represent r^2 measurements. The middle panel shows the four common haplotypes, H1, H2, H3 and H4, and their respective frequencies. The mean warfarin dose ± 95% confidence interval (CI) associated with each haplotype is shown on the right-hand side.

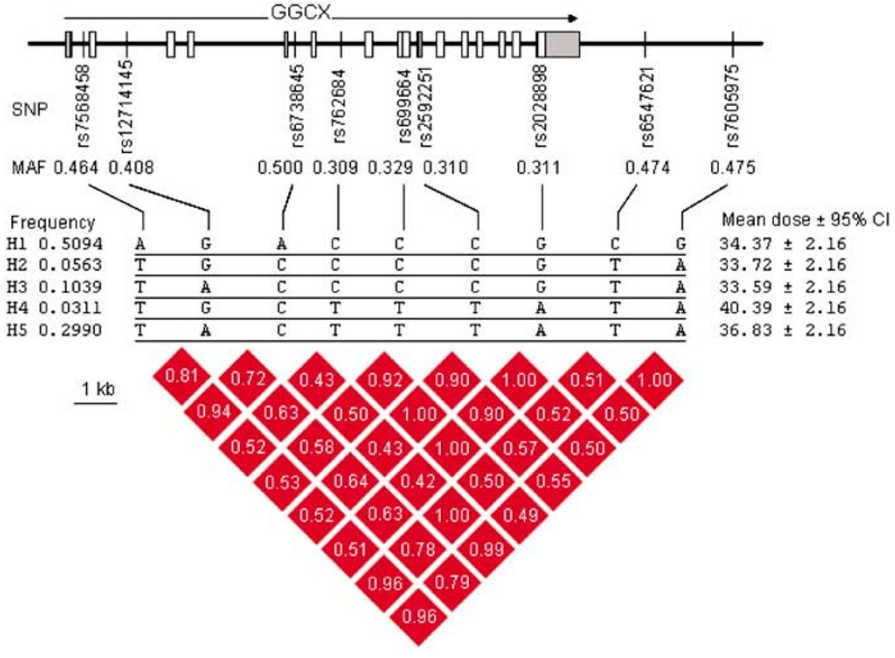

Figure 3 Genomic organisation, LD and common haplotypes across the *GGCX* gene. Shaded and open rectangles indicate the UTR and coding parts of exons, respectively. The SNP positions along chromosome 2 are rs7568458, 85699833 bp; rs12714145, 85698999 bp; rs6738645, 85694786 bp; rs762684, 85693681 bp; rs699664, 85692194 bp; rs2592551, 85691789 bp; rs2028898, 85688928 bp; rs6547621, 85686334 bp and rs7605975, 85684206 bp (NCBI 35). The MAF of each SNP is given, with pairwise LD displayed in red (strong LD) and light blue (low LD) rectangles; values represent r^2 measurements. The middle panel shows the five common haplotypes and their respective frequencies. The mean warfarin dose ± 95% CI associated with each haplotype is shown on the right-hand side.

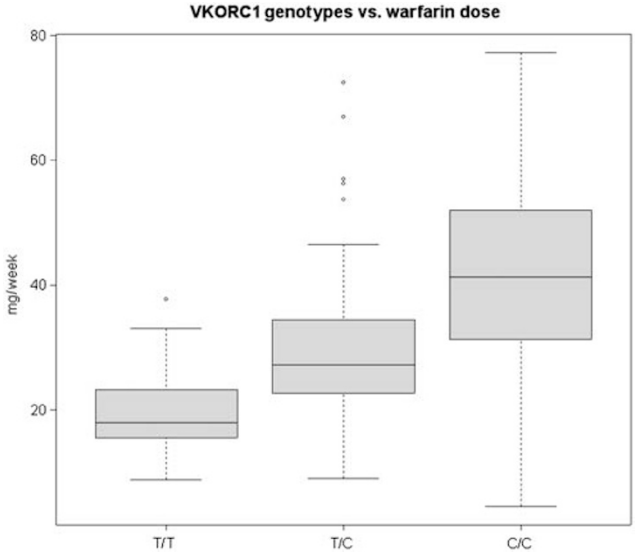

Figure 4 Box and whisker plot of mean weekly warfarin doses for different *VKORC1* genotypes (SNP rs2359612). The horizontal line indicates the median, the box covers the 25–75% percentiles and the maximum length of each whisker is 1.5 times the interquartile range. Points outside this show up as outliers. In all, 200 individuals were genotyped for rs2359612; genotyping failed in one individual for technical reasons. The maintenance dose of warfarin was significantly related to all the five studied *VKORC1* SNPs.

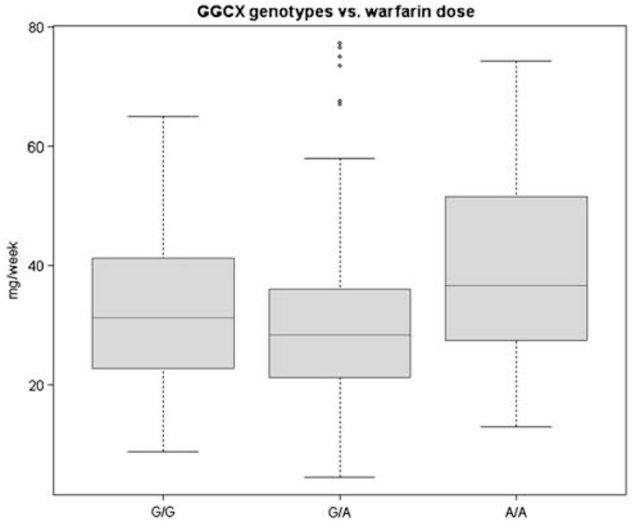

Figure 5 Box and whisker plot of mean weekly warfarin doses for different *GGCX* genotypes (SNP rs12714145). The horizontal line indicates the median, the box covers the 25–75% percentiles and the maximum length of each whisker is 1.5 times the interquartile range. Points outside this show up as outliers. In all, 200 individuals were genotyped for rs12714145; genotyping failed in one individual for technical reasons. The maintenance dose of warfarin was significantly related to *GGCX* SNP rs12714145.

Table 1 Univariate regression models for warfarin maintenance dose and dose/bodyweight

Variables	*Dose P*	r^2	*Dose/BW P*	r^2
VKORC1	<0.0001	0.285	<0.0001	0.270
GGCX	0.0360	0.033	0.4223	0.009
CYP2C9	0.0003	0.112	0.0008	0.105
Age	<0.0001	0.095	0.0002	0.072
Bodyweight	0.0018	0.049	0.0034	0.044
Interaction	0.0239	0.037	0.0076	0.050
Gender	0.0314	0.023	0.3467	0.005
Indication	0.0819	0.015	0.0002	0.069
PT INR	0.1272	0.012	0.0042	0.042

Concomitant medication is divided into three groups: drugs with no interaction, drugs that potentiate and drugs that decrease the effect of warfarin. Indication for treatment is divided into two groups: heart valve prosthesis and other indications. The factors *VKORC1* SNP rs2359612, *CYP2C9* variants **2* and **3*, age, bodyweight (BW), concomitant medication, gender, the indication for treatment and PT INR value are tested for covariance with warfarin maintenance dose and dose/bodyweight using univariate analysis in SAS. The explanatory value of *VKORC1* SNP rs9923231 was still higher, $r^2=0.30$ for dose and $r^2=0.27$ for dose/BW, based on 174 genotyped patients.

univariate model used, rs2359612 was a better predictor of warfarin maintenance dose than rs7294; addition of rs7294 to the model increased the explanatory value by only 3%. Note that rs9923231, rs9934438 and rs2359612 have pairwise r^2 values close to 1, implying that their genotypes are in near perfect concordance. In contrast to *VKORC1*, only one of the studied *GGCX* SNPs reaches statistical significance, rs12714145 (intron 2), $P=0.0360$ (Figure 5). *GGCX* SNPs rs762684 (intron 6) and rs2592551 (exon 9) also show a tendency towards association with warfarin dose ($P=0.0613$ and 0.0870).

The mean warfarin dose associated with each *VKORC1* and *GGCX* haplotype was calculated (Figures 2 and 3). A global test for statistical difference among the haplotype means revealed a highly significant difference among *VKORC1* haplotypes (global $P \leqslant 4.73 \times 10^{-9}$), but not among *GGCX* haplotypes (global $P=0.757$). To characterise warfarin dose differences among *VKORC1* haplotypes, the means for each pair of haplotypes were statistically compared (Table 2). Haplotypes that share alleles at the first three SNPs (rs9923231, rs9934438 and rs2359612) do not exhibit significant differences in warfarin dose; G–C–C haplotypes H1 *vs* H2 ($P=0.09$), and A–T–T haplotypes H3 *vs* H4 ($P=0.54$). However, every pair of haplotypes with different alleles at the first three SNPs (G–C–C *vs* A–T–T) shows significant differences ($P=1.26 \times 10^{-9}$ to 0.0163). Figure 2 and Table 2 both illustrate that G–C–C haplotypes require significantly higher mean doses (35.24–40.15 mg) than A–T–T haplotypes (23.86–26.41 mg). These results, and the results from the univariate model above, indicate that the first three SNPs are the best predictors of warfarin dose, and that rs7294 and rs11150606 provide much less additional predictive information.

To increase the power to predict variable warfarin sensitivity, we combined *VKORC1* genotyping results with other genetic and nongenetic factors. Polymorphisms in the liver enzyme CYP2C9, which metabolises warfarin, are known to be associated with warfarin sensitivity.[1–3,5,11,20–24] Our subjects have previously been genotyped for *CYP2C9*, and the frequency of CYP2C9 homozygous extensive metabolisers was 66.7%, heterozygous extensive metabolisers 31.3% and poor metabolisers 2.0%.[3] The combined effect of *VKORC1* and *CYP2C9* on warfarin dose is presented in Figure 6, which shows that *VKORC1* has a clear effect on all extensive metabolisers. In a multiple model, *VKORC1*, *CYP2C9*, age, bodyweight, interacting drugs, and indication for treatment together account for 56.0% of the total inter-individual variance in warfarin response (Table 3). The explanatory value increases marginally to 57.4%, when *GGCX* is added to the model.

Table 2 Statistical comparison of the means for each pair of *VKORC1* haplotypes

Haplotypes	*H1*	*H2*	*H3*	*H4*
H1	—	0.0913005	1.26×10^{-9}	0.00145198
H2		—	0.000117041	0.0163264
H3			—	0.540107
H4				—

Haplotypes that share alleles at the first three SNPs do not exhibit significant differences in warfarin dose. Every haplotype with different alleles at the first three SNPs shows significant differences, G–C–C haplotypes H1 and H2 requiring significantly higher mean doses (40.15 and 35.24 mg) than A–T–T haplotypes H3 and H4 (26.41 and 23.86 mg).

DISCUSSION

Warfarin acts by interfering with VKOR, which catalyses an essential step in the activation of several blood-clotting

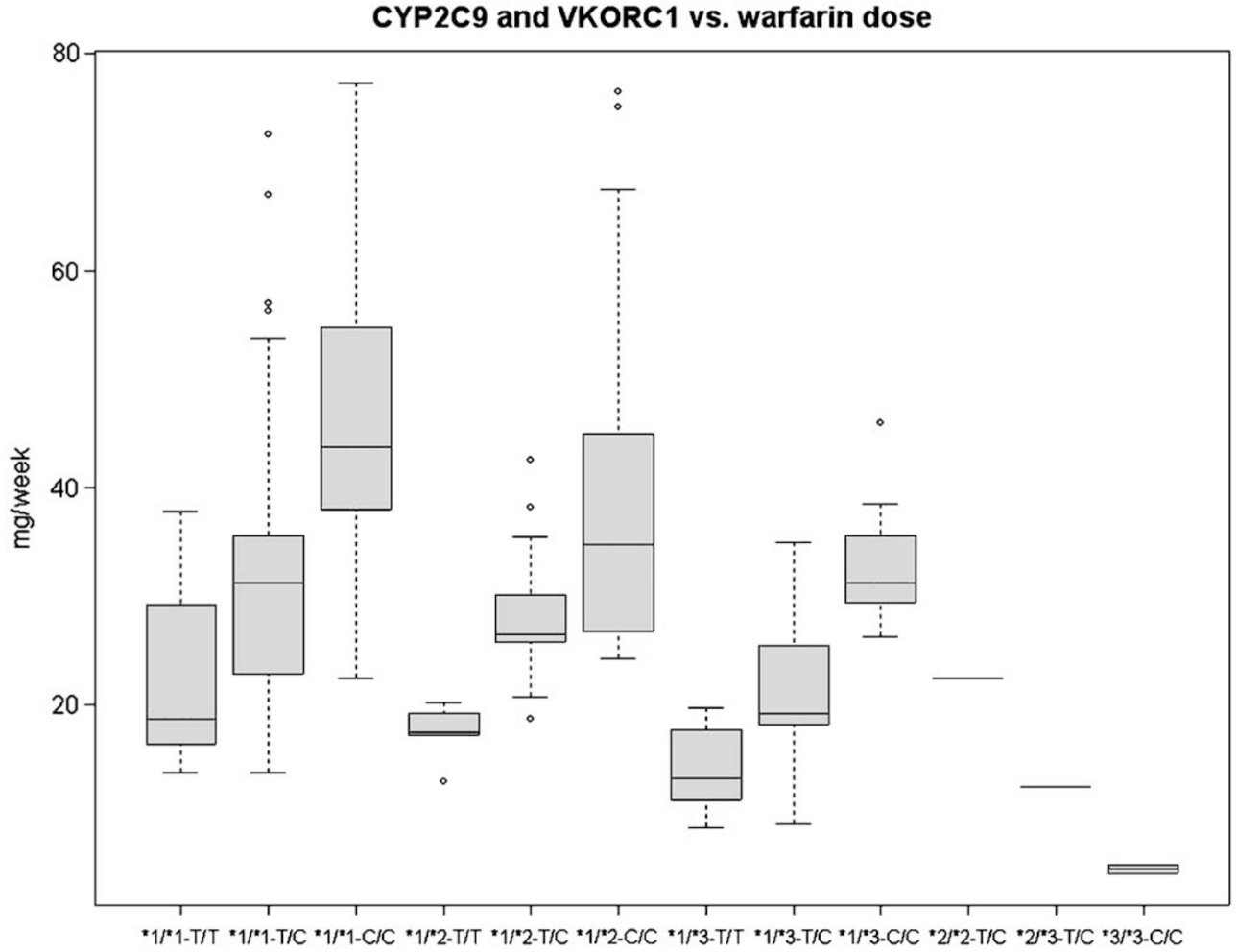

Figure 6 Box and whisker plot of mean weekly warfarin doses for different genotypes of *VKORC1* (SNP rs2359612) and *CYP2C9* (alleles **1*, **2*, **3*). The horizontal line indicates the median, the box covers the 25–75% percentiles and the maximum length of each whisker is 1.5 times the interquartile range. Points outside this show up as outliers. Maintenance dose of warfarin was significantly related to *VKORC1* and *CYP2C9* genotypes.

Table 3 Multiple regression model for warfarin maintenance dose

Variables	*Dose P*
VKORC1	<0.0001
CYP2C9	<0.0001
Age	<0.0001
Bodyweight	<0.0001
Interaction	0.0006
Indication	0.0140

Total r^2 for the model = 0.5605.
Using *VKORC1* and *CYP2C9* genotypes, age, bodyweight, interacting drugs and indication for treatment in a multiple model, we account for 56.0% of the variance in warfarin maintenance dose. When *GGCX* is added to the model, the explanatory value increases to 57.4%.

proteins. The discovery of rare mutations in *VKORC1* and *GGCX* causing deficiency in vitamin K-dependent clotting factors, and for *VKORC1* also hereditary warfarin resistance were presented last year.[31,34] This led us to test the hypothesis that common polymorphisms in these genes affect warfarin response in the general population.

It is well known that polymorphisms in *CYP2C9* are associated with warfarin sensitivity.[3] An Italian group lead by Margaglione recently described an association between warfarin dose and genotypes of *CYP2C9* and two non-coding *VKORC1* SNPs (rs9934438 and rs7294) in 147 patients.[41] In their study, *CYP2C9* and *VKORC1* together account for 35% of interindividual variability. In our material, *CYP2C9* and *VKORC1* together explain 40% of variance in dose, and *VKORC1* is the better predictor of the two. In contrast, in the Italian study, *CYP2C9* has a larger impact on dose than *VKORC1*. This discrepancy could be due to the fact that *CYP2C9* variant alleles are more frequent in the Italian population than in the Swedish.[1,41,42] In Margaglione's study, the allele frequencies of *CYP2C9*2* and **3* were 17.0% and 8.8%, respectively,[41] as compared with our figures of 11.2% and 6.5%.[3] On the other hand, there was no major difference between populations in the frequency of the intragenic *VKORC1* SNP rs9934438: MAF 39.8% in the Italians, and 38.8% in the Swedes.[41]

All the studied *VKORC1* polymorphisms are non-coding, and do not change the molecular structure of the enzyme. The observed association between polymorphisms and dose variation could instead be due to one of the alleles affecting mRNA transcription, splicing or stability. This effect may be mediated by one of the analysed SNPs or by an unidentified causal variant in LD with the alleles studied here.

A Japanese group reported an association between genetic variants in *GGCX* and warfarin dose.[43] They showed that warfarin dose increased with the number of microsatellite (CAA)n repeats in intron 6. Interestingly, the *GGCX* SNP in intron 6 shows the same trend, but in our material a SNP in intron 2 was the best predictor of warfarin dose. In fact, all the tested GGCX SNPs are in high LD, and it is therefore not surprising that several SNPs show the same trend.

Knowledge of biochemical mechanisms, site of drug action and the human genome enables discovery of new genetic factors that cause variable drug response. By combining genotyping with accurate clinical measurements, it should be possible to develop an effective algorithm for individualised drug treatment using genetic and clinical factors. Already, more than half the variance in maintenance warfarin dose is explained by *VKORC1*, *CYP2C9* and four patient characteristics. This compares favourably with the recently published value of 39% that was obtained using a predictive dosage algorithm based on *CYP2C9* and seven clinical and demographic factors.[44] Verification of our findings and continued search for additional factors will be performed in a larger patient cohort. These results will eventually enable prediction of individualised dosage in the initiation phase of warfarin therapy, and minimise the risk of early haemorrhage without compromising anticoagulant effect. This strategy may also be used to identify warfarin-sensitive patients, who require one of the expensive novel anticoagulants. Implementation of personalised warfarin treatment would therefore be both beneficial and cost-effective.

MATERIALS AND METHODS

Patients

This study was approved by the local Ethics Committee. After informed consent, we enrolled 201 patients that were treated with warfarin at the anticoagulation clinic at Uppsala University Hospital.[3] The patients were essentially Caucasian; 194 being of Swedish origin, four of other European descent and three from the Middle East. When the patients were recruited in 2000, they were 28–88 years old (Table 1). They had been treated with warfarin for at least 2 months (range 2.4 months–26 years, median 2 years). At six consecutive visits, five weekly warfarin doses and five corresponding PT INR values were registered. Individual warfarin dose requirement ranged from 4.5 to 77.25 mg/week. Information about age, gender, bodyweight (missing in seven patients), other diseases and indication for treatment was taken from the patients' medical records (Table 4). Patients were stratified into two treatment groups: patients with heart valve prosthesis, where a higher target INR usually is recommended and patients treated for other indications. Concurrent medications were registered, and drugs were classified as interacting if they had moderate or major interactions with warfarin according to the database MICROMEDEX® Healthcare Series (http://www.micromedex.com/ in May 2002). The patients had a total of 107 concurrent medications known to influence warfarin (Table 4). Patients were divided into three groups: individuals with drugs that lower the effect of warfarin by inducing its metabolism ($n = 4$), those with medications that potentiate the effect of warfarin ($n = 74$) and patients without any known interactions ($n = 123$). Whole blood was collected from all patients, and DNA was extracted using standard procedures.

Table 4 Characteristics of patients in the dose requirement study ($n = 201$)

Characteristics	*Patients (%)*
Indication	
Atrial fibrillation	113 (56.2)
Heart valve prosthesis	49 (24.4)
Deep vein thrombosis/pulmonary embolus	9 (4.5)
Cardiomyopathy	8 (4.0)
Transischemic attack	5 (2.5)
Other diseases	
Hypertension	78 (38.8)
Heart failure	51 (25.4)
Angina pectoris	35 (17.4)
Type 2 diabetes mellitus	18 (9.0)
Interacting medication	
Simvastatin ↗	25 (12.4)
Aspirin ↗	21 (10.4)
Paracetamol ↗	18 (9.0)
Amiodarone ↗	9 (4.5)
Disopyramide ↗	7 (3.5)
Dextropropoxyphene ↗	7 (3.5)
Propafenone ↗	3 (1.5)
Carbamazepine ↘	3 (1.5)
Nonsteroidal anti-inflammatory drug ↗	2 (1.0)
Phenytoin ↘	1 (0.5)
Mianserin ↘	1 (0.5)
Gender	
Men	135 (67.2)
Women	66 (32.8)
Age	
Mean years (range)	66.9 (28–88)

A patient can have more than one indication, concurrent disease and interacting medication, and only the most common ones are shown. Drugs potentiating the effect of warfarin are indicated by ↗ and drugs decreasing its effect by ↘.

Genotyping

SNP sequences were downloaded from the dbSNP database (build 121) and assays were designed with the SpectroDESIGNER™ software. SNP typing was performed using the MassARRAY™ platform (Sequenom, Hamburg, Germany). Primer sequences are listed in Table 5. Polymerase chain reaction (PCR) amplification was performed in 5-μl reactions using 3.5 ng DNA and 150 nM of each forward and reverse primer, 200 μM deoxynucleotide triphosphates (dNTPs), $1 \times$ PCR buffer and 0.04 U Titanium® polymerase (BD BioSciences, Clontech, CA, USA). Cycling conditions were 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min, and then 72°C for 3 min. Primer extension, sample clean-up and MALDI-TOF mass spectrometry analysis were performed as described by Whittaker *et al*.[45]

Genotyping was carried out at a multiplex level of four SNPs per well and data quality was assessed by duplicate DNAs ($n = 4$). SNPs with more than one discrepant call were

Table 5 PCR primers and extend primers for the genotyping reactions

	Forward primer	*Reverse primer*	*Extend Primer*	*Term*
VKORC1				
rs9923231	ACGTTGGATGAAGACGCTAGACCCAATGGT	ACGTTGGATGGAGAAGACCTGAAAAACAACC	GGCGTGAGCCACCGCACC	ACG
rs9934438	ACGTTGGATGTGACATGGAATCCTGACGTG	ACGTTGGATGATTTCCAAGAAGCCACCTGG	GTGCCAGGAGATCATCGAC	ACG
rs2359612	ACGTTGGATGAAATCCGGCCAAGTCTGAACC	ACGTTGGATGAGCTCCAGAGAAGGCATCAC	ATGTGTCAGCCAGGACC	ACT
rs7294	ACGTTGGATGGGTGTAAAAAAGAGCCGAGCG	ACGTTGGATGTTCTAGATTACCCCCTCCTC	ACATTTGGTCCATTGTCATGTG	ACG
rs11150606	ACGTTGGATGGGACAGGTATCTGCTGTCAG	ACGTTGGATGTGTTGCCCTCCTGAGGCTTG	TTCCACAGCCTGTGGAC	ACT
GGCX				
rs7568458	ACGTTGGATGTTGAGGCAACCTCATTGAGC	ACGTTGGATGGGCTCCACCTCAAATCAAAG	GCTCTGAGCTGTTGGTGC	CGT
rs12714145	ACGTTGGATGTGAAGTTGCCCAGAAGACTC	ACGTTGGATGCTTGATTAGGGAGTCACAGC	CAGAAGACTCAGAGAAACA	ACT
rs6738645	ACGTTGGATGTAGCAGGGACAAAGCTCTAG	ACGTTGGATGTTTGCTGGAAAGCTAGGCTG	TAGTCTTTCCTTGACAAATAAGA	ACT
rs762684	ACGTTGGATGCCTTTTAAAGTGTCCTCTCAC	ACGTTGGATGTGAGATGTGTGTGTTGTG	AAGTGTCCTCTCACTCATGAC	ACG
rs699664	ACGTTGGATGTTGAGGGGCAACAGTTGTTG	ACGTTGGATGTGTTCTCCTACGTCATGCTG	CAACAGTTGTTGCAACCTT	ACG
rs2592551	ACGTTGGATGTAGGTGATCTTCACGTGCTG	ACGTTGGATGATGGGCTGTATGGCTATTCC	TCTTCACGTGCTGGTGGGA	ACT
rs2028898	ACGTTGGATGTACAAGTCATCAGGAAGCTG	ACGTTGGATGTTCTTCTGGTGAAACAGGAG	GAAGCTGAAAAACAGGAAAAAGCC	ACT
rs6547621	ACGTTGGATGGAGAGGTGGTATACTAACAG	ACGTTGGATGTGACTGGTAACAAAAGTCCC	TTCAAGTGTCTTCTCCCAA	ACT
rs7605975	ACGTTGGATGCATTTGTTAAAGTGCTTTGGG	ACGTTGGATGCAGGTTATTCATATGATAGC	CATTATCAAGCCACTGCACTC	ACG

The extension is terminated by three ddNTPs. The fourth nucleotide is added as an ordinary dNTP.

removed. Finally, we removed loci with results for less than 80% of individuals or zero heterozygosity, and we flagged markers out that were not in Hardy–Weinberg equilibrium ($\chi^2 \geqslant 10$).

Statistical Analysis

We assessed the quality of the genotype data by testing for Hardy–Weinberg equilibrium. Univariate and multiple analyses of predictor's impact on warfarin dose were calculated using linear regression models (as implemented by SAS software). The QTPhase component of Unphased software was used to estimate haplotype frequencies, calculate means and variances of warfarin dose associated with each haplotype, and statistically test for differences among the haplotype means.[46] Pairwise LD was quantified by the standard r^2 measure.[47]

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DUALITY OF INTEREST

None declared.

ABBREVIATIONS

CYP2C9	*cytochrome P450 2C9*
ddNTP	*dideoxynucleotide triphosphate*
dNTP	*deoxynucleotide triphosphate*
GGCX	*gamma-glutamyl carboxylase*
LD	*linkage disequilibrium*
PCR	*polymerase chain reaction*
PT INR	*prothrombin time international normalised ratio*
UTR	*untranslated region*
VKOR	*vitamin K epoxide reductase*
VKORC1	*vitamin K epoxide reductase complex subunit 1*

REFERENCES

1 Scordo MG, Pengo V, Spina E, Dahl ML, Gusella M, Padrini R. Influence of CYP2C9 and CYP2C19 genetic polymorphisms on warfarin maintenance dose and metabolic clearance. *Clin Pharmacol Ther* 2002; **72**: 702–710.
2 Aithal G, Day C, Kesteven P, Daly A. Association of polymorphisms in the cytochrome *P*450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet* 1999; **353**: 689–717.
3 Wadelius M, Sörlin K, Wallerman O, Karlsson J, Yue QY, Magnusson PK *et al.* Warfarin sensitivity related to CYP2C9, CYP3A5, ABCB1 (MDR1) and other factors. *Pharmacogenomics J* 2004; **4**: 40–48.
4 van den Besselaar AM. Standardization of the prothrombin time in oral anticoagulant control. *Haemostasis* 1985; **15**: 271–277.
5 Takahashi H, Echizen H. Pharmacogenetics of CYP2C9 and interindividual variability in anticoagulant response to warfarin. *Pharmacogenomics J* 2003; **3**: 202–214.
6 Landefeld C, Beyth R. Anticoagulant-related bleeding: clinical epidemiology, prediction and prevention. *Am J Med* 1993; **95**: 315–328.
7 Mathiesen T, Benediktsdottir K, Johnsson H, Lindqvist M, von Holst H. Intracranial traumatic and non-traumatic haemorrhagic complications of warfarin treatment. *Acta Neurol Scand* 1995; **91**: 208–214.
8 Levine M, Raskob G, Landefeld S, Kearon C. Hemorrhagic complications of anticoagulant treatment. *Chest* 1998; **114**: 511S–523S.
9 Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ *et al.* Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *BMJ* 2004; **329**: 15–19.
10 Runciman WB, Roughead EE, Semple SJ, Adams RJ. Adverse drug events and medication errors in Australia. *Int J Qual Health Care* 2003; **15**(Suppl 1): i49–59.
11 Loebstein R, Yonath H, Peleg D, Almog S, Rotenberg M, Lubetsky A *et al.* Interindividual variability in sensitivity to warfarin—nature or nurture? *Clin Pharmacol Ther* 2001; **70**: 159–164.
12 Gage BF, Eby CS. Pharmacogenetics and anticoagulant therapy. *J Thromb Thrombolysis* 2003; **16**: 73–78.
13 Kamali F, Khan TI, King BP, Frearson R, Kesteven P, Wood P *et al.* Contribution of age, body size, and CYP2C9 genotype to anticoagulant response to warfarin. *Clin Pharmacol Ther* 2004; **75**: 204–212.
14 Hillman MA, Wilke RA, Caldwell MD, Berg RL, Glurich I, Burmester JK. Relative impact of covariates in prescribing warfarin according to CYP2C9 genotype. *Pharmacogenetics* 2004; **14**: 539–547.
15 Daly AK, King BP. Pharmacogenetics of oral anticoagulants. *Pharmacogenetics* 2003; **13**: 247–252.
16 Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T *et al.* Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin–drug interactions. *Chem Res Toxicol* 1992; **5**: 54–59.
17 Takahashi H, Echizen H. Pharmacogenetics of warfarin elimination and its clinical implications. *Clin Pharmacokinet* 2001; **40**: 587–603.
18 Kaminsky L, Zhang Z. Human *P*450 metabolism of warfarin. *Pharmacol Ther* 1997; **73**: 67–74.
19 Xie HG, Prasad HC, Kim RB, Stein CM. CYP2C9 allelic variants: ethnic distribution and functional significance. *Adv Drug Deliv Rev* 2002; **54**: 1257–1270.
20 Furuya H, Fernandez-Salguero P, Gregory W, Taber H, Steward A, Gonzalez FJ *et al.* Genetic polymorphism of CYP2C9 and its effect on warfarin maintenance dose requirement in patients undergoing anticoagulation therapy. *Pharmacogenetics* 1995; **5**: 389–392.
21 Margaglione M, Colaizzo D, D'Andrea G, Brancaccio V, Ciampa A, Grandone E *et al.* Genetic modulation of oral anticoagulation with warfarin. *Thromb Haemost* 2000; **84**: 775–778.
22 Tabrizi AR, Zehnbauer BA, Borecki IB, McGrath SD, Buchman TG, Freeman BD. The frequency and effects of cytochrome *P*450 (CYP) 2C9 polymorphisms in patients receiving warfarin. *J Am Coll Surg* 2002; **194**: 267–273.
23 Taube J, Halsall D, Baglin T. Influence of cytochrome P-450 CYP2C9 polymorphisms on warfarin sensitivity and risk of over-anticoagulation in patients on long-term treatment. *Blood* 2000; **96**: 1816–1819.
24 Higashi M, Veenstra D, Kondo L, Wittkowsky A, Srinouanprachanh S, Farin F *et al.* Association between CYP 2C9 genetic variants and anticoagulation-related outcomes during warfarin treatment. *JAMA* 2002; **287**: 1690–1698.
25 Sadler JE. Medicine: K is for koagulation. *Nature* 2004; **427**: 493–494.
26 Linder MW. Genetic mechanisms for hypersensitivity and resistance to the anticoagulant Warfarin. *Clin Chim Acta* 2001; **308**: 9–15.
27 Bell RG. Metabolism of vitamin K and prothrombin synthesis: anticoagulants and the vitamin K–epoxide cycle. *Fed Proc* 1978; **37**: 2599–2604.
28 Bell RG, Sadowski JA, Matschiner JT. Mechanism of action of warfarin. Warfarin and metabolism of vitamin K 1. *Biochemistry* 1972; **11**: 1959–1961.
29 Begent LA, Hill AP, Steventon GB, Hutt AJ, Pallister CJ, Cowell DC. Characterization and purification of the vitamin K1 2,3 epoxide reductases system from rat liver. *J Pharm Pharmacol* 2001; **53**: 481–486.

30 Fregin A, Rost S, Wolz W, Krebsova A, Muller CR, Oldenburg J. Homozygosity mapping of a second gene locus for hereditary combined deficiency of vitamin K-dependent clotting factors to the centromeric region of chromosome 16. *Blood* 2002; **100**: 3229–3232.
31 Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ *et al.* Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004; **427**: 537–541.
32 Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature* 2004; **427**: 541–544.
33 Harrington DJ, Underwood S, Morse C, Shearer MJ, Tuddenham EG, Mumford AD. Pharmacodynamic resistance to warfarin associated with a Val66Met substitution in vitamin K epoxide reductase complex subunit 1. *Thromb Haemost* 2005; **93**: 23–26.
34 Rost S, Fregin A, Koch D, Compes M, Muller CR, Oldenburg J. Compound heterozygous mutations in the gamma-glutamyl carboxylase gene cause combined deficiency of all vitamin K-dependent blood coagulation factors. *Br J Haematol* 2004; **126**: 546–549.
35 Suttie JW, Canfield LM, Shah DV. Microsomal vitamin K-dependent carboxylase. *Methods Enzymol* 1980; **67**: 180–185.
36 Berkner KL, McNally BA. Purification of vitamin K-dependent carboxylase from cultured cells. *Methods Enzymol* 1997; **282**: 313–333.
37 Lingenfelter SE, Berkner KL. Isolation of the human gamma-carboxylase and a gamma-carboxylase-associated protein from factor IX-expressing mammalian cells. *Biochemistry* 1996; **35**: 8234–8243.
38 Kuo WL, Stafford DW, Cruces J, Gray J, Solera J. Chromosomal localization of the gamma-glutamyl carboxylase gene at 2p12. *Genomics* 1995; **25**: 746–748.
39 Wu SM, Stafford DW, Frazier LD, Fu YY, High KA, Chu K *et al.* Genomic sequence and transcription start site for the human gamma-glutamyl carboxylase. *Blood* 1997; **89**: 4058–4062.
40 Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B *et al.* The structure of haplotype blocks in the human genome. *Science* 2002; **296**: 2225–2229.
41 D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V *et al.* A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood* 2005; **105**: 645–649.
42 Yasar U, Elisson E, Dahl M, Johansson I, Ingelman-Sundberg M, Sjökvist F. Validation of methods for CYP2C9 genotyping: frequencies of mutant alleles in a Swedish population. *Biochem Biophys Res Commun* 1999; **254**: 628–631.
43 Shikata E, Ieiri I, Ishiguro S, Aono H, Inoue K, Koide T *et al.* Association of pharmacokinetic (CYP2C9) and pharmacodynamic (factors II, VII, IX, and X; proteins S and C; and gamma-glutamyl carboxylase) gene variants with warfarin sensitivity. *Blood* 2004; **103**: 2630–2635.
44 Gage BF, Eby C, Milligan PE, Banet GA, Duncan JR, McLeod HL. Use of pharmacogenetics and clinical factors to predict the maintenance dose of warfarin. *Thromb Haemost* 2004; **91**: 87–94.
45 Whittaker P, Bumpstead S, Downes K, Ghori J, Deloukas P. SNP analysis by MALDI-TOF mass spectrometry. In: Celis J, Carter N, Simons K, Small JV, Hunter T, Shotton D (eds). 3rd ed. *Cell Biology: A Laboratory Handbook*. Amsterdam: Elsevier, 2005.
46 Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 2003; **25**: 115–121.
47 Pritchard JK, Przeworski M. Linkage disequilibrium in humans: models and data. *Am J Hum Genet* 2001; **69**: 1–14.