

# Analysis of *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms in a population from South-Eastern Europe

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## Abstract

The CYP2C9 enzyme metabolizes a wide range of relevant drugs, among which are oral anticoagulants. VKORC1 is the pharmacodynamic target of the oral anticoagulants. The genetic polymorphisms *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A are the major determinants of the inter-individual variability in the dosage requirements of oral anticoagulants. This study provides a first evaluation of these 3 polymorphisms in a Romanian population. A total of 332 Romanian individuals were genotyped for the *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms using the PCR-RFLP technique. Sixty-two individuals (18.7%) were heterozygous for *CYP2C9\*2*, whereas 47 individuals (14.1%) were heterozygous for *CYP2C9\*3*. Fourteen individuals (4.2%) had a *CYP2C9\*2* homozygous, *CYP2C9\*3* homozygous or *CYP2C9\*2/CYP2C9\*3* compound heterozygous genotype. These individuals are predicted to have the lowest CYP2C9 enzymatic activity. The allele frequencies of the *CYP2C9\*2* and *CYP2C9\*3* polymorphisms were 11.3% and 9.3% respectively. For the *VKORC1* -1639 G>A polymorphism, there were 170 heterozygotes (51.2%) and 55 (16.6%) homozygotes for the A allele. The frequency of the A allele was 42.2%. Overall, the distribution of the *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms observed in our cohort is in accordance with other Caucasian populations. A large number of Romanians are expected to harbour at least one *CYP2C9* variant allele and/or one *VKORC1* -1639 G>A allele. This frequency has major implications in the pharmacogenomics of oral anticoagulants in Romanians.

**Keywords:** CYP2C9 • VKORC1 • Romanians • oral anticoagulants

## Introduction

The CYP2C subfamily of cytochrome P450 comprises 4 members: CYP2C8, CYP2C9, CYP2C18 and CYP2C19. Among them, CYP2C9 is the most abundant isoform in the liver, metabolizing a wide range of clinically relevant drugs, such as oral anticoagulants (warfarin, acenocoumarol and phenprocoumon), antidiabetic drugs (tolbutamide and glipizide), the anti-convulsant phenytoin, and a wide range of non-steroidal anti-inflammatory drugs (e.g. diclofenac, ibuprofen and piroxicam) [1, 2]. The gene encoding the CYP2C9 enzyme is polymorphic. Several variants of the *CYP2C9* gene

have been described, but the most prevalent and most studied of them are the *CYP2C9\*2* and *CYP2C9\*3* polymorphisms. The *CYP2C9\*2* allele is the result of a C>T transition in position 430 of the *CYP2C9* gene, leading to an Arg-to-Cys substitution at residue 144 of the CYP2C9 molecule. The *CYP2C9\*3* allele is the result of an A>T transversion in position 1075 of the *CYP2C9* gene, leading to an Ile-to-Leu substitution at residue 359 of the CYP2C9 molecule [3]. Both alleles lead to a significant reduction in the enzymatic activity of the CYP2C9 molecule, representing the major causes of decreased CYP2C9 enzymatic activity, at least in Caucasians [3].

Vitamin K epoxide reductase catalyses the transformation of vitamin K 2,3-epoxide into vitamin K hydroxyquinone, which is essential for the synthesis of factors II, VII, IX and XI of the coagulation system, as well as proteins C and S. The enzyme vitamin K epoxide reductase, which is the pharmacodynamic target of oral anticoagulants, is encoded by the *VKORC1* gene. While rare mutations of this gene produce clotting factor deficiencies or warfarin resistance, common

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functional polymorphisms are associated with variations in the dosage requirement of oral anticoagulants [4]. Two common polymorphisms of the *VKORC1* gene, namely -1639 G>A and 1173 C>T, which are in complete linkage disequilibrium, were shown to confer a high risk towards over-anticoagulation, predisposing the individuals carrying these polymorphisms to haemorrhagic incidents [5].

The distribution of the *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms has been extensively assessed in many populations; however, data for the Romanian population are lacking. Here, we analysed the frequency of these polymorphisms in a cohort of Romanians and compared it with similar data from other populations.

## Materials and methods

### Study participants

Between 2007 and 2010, a total of 332 individuals were enrolled in this study. All participants originated from the Transylvania region (North-Western and central parts of Romania). The study group comprised 155 women (46.7%) and 177 men (53.3%). The median age of the study participants was 69 years (range 34–83 years). Written and informed consent was obtained from all the participants prior to their enrolment. This study was reviewed and approved by the Ethics Committee of the “Iuliu Hațieganu” University of Medicine and Pharmacy of Cluj-Napoca. After admission to the study, 3 ml of blood was collected from each patient into an EDTA tube, and the genomic DNA was obtained using a commercially available procedure (Wizard Genomic DNA Purification Kit; Promega, Madison, WI, USA).

### Genotyping for the *CYP2C9\*2* and *CYP2C9\*3* polymorphisms

Genotyping for the *CYP2C9\*2* and *CYP2C9\*3* polymorphisms was performed with PCR-RFLP (polymerase chain reaction–restriction fragment length polymorphism), essentially as previously described [6]. Briefly, a 372-bp amplicon was obtained by PCR to study the *CYP2C9\*2* allele. The amplicon was digested overnight with the *Sau96I* restriction enzyme (Fermentas MBI, Vilnius, Lithuania), giving rise to 3 fragments with lengths of 179, 119 and 74 bp in the case of the wild-type allele. However, the *CYP2C9\*2* allele abolishes one restriction site of the *Sau96I* enzyme, giving rise to only 2 fragments, with lengths of 253 and 119 bp. To analyse the *CYP2C9\*3* variant, a 130-bp amplicon was obtained by PCR. This amplicon was digested overnight with the *StyI* restriction enzyme (Fermentas MBI), and the wild-type allele was resistant to the *StyI* digestion. However, the *CYP2C9\*3* allele creates a restriction site for *StyI*, cutting the 130-bp amplicon into 2 fragments, with lengths of 104 and 26 bp.

### Genotyping for the *VKORC1* -1639 G>A polymorphism

Genotyping for the *VKORC1* -1639 G>A polymorphism was performed with PCR-RFLP, essentially as previously described [7]. Briefly, a 290-bp

amplicon was obtained by PCR. The amplicon was digested overnight with *MspI* restriction enzyme (Fermentas MBI). If the G allele was present, then the 290-bp amplicon was cut in 2 fragments, with lengths of 167 and 123 bp, whereas the presence of the A allele rendered the amplicon resistant to digestion with the *MspI* restriction enzyme.

### Statistical analysis

The distribution of the *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms was compared between Romanians and other populations by Fisher's exact test, using the GraphPad InStat version 3.0 statistical software (GraphPad, San Diego, CA, USA). The level of statistical significance was set at 0.05.

## Results

### *CYP2C9\*2* and *CYP2C9\*3*

Sixty-two individuals (18.7%) were heterozygous for *CYP2C9\*2*, whereas 47 individuals (14.1%) were heterozygous for *CYP2C9\*3*. Two individuals (0.6%) were homozygous for *CYP2C9\*2*, whereas 3 individuals (0.9%) were homozygous for *CYP2C9\*3*. A total number of 9 individuals (2.7%) were *CYP2C9\*2/CYP2C9\*3* compound heterozygotes. Thus, 14 individuals (4.2%) from the cohort analysed are predicted to have the lowest CYP2C9 enzymatic activity, with respect to the *CYP2C9\*2* and *CYP2C9\*3*. Overall, the *CYP2C9\*2* allele had a frequency of 11.3%, whereas the *CYP2C9\*3* allele had a frequency of 9.3%. The observed genotype frequencies for both

**Table 1** Genotype and alleles frequencies observed for the *CYP2C9* polymorphisms in our study

| Genotype distribution (N = 332 individuals) |                  |
|---|------------------|
| Genotype                                    | Frequency; n (%) |
| <i>CYP2C9*1/CYP2C9*1</i>                    | 209 (63)         |
| <i>CYP2C9*1/CYP2C9*2</i>                    | 62 (18.7)        |
| <i>CYP2C9*1/CYP2C9*3</i>                    | 47 (14.1)        |
| <i>CYP2C9*2/CYP2C9*2</i>                    | 2 (0.6)          |
| <i>CYP2C9*2/CYP2C9*3</i>                    | 9 (2.7)          |
| <i>CYP2C9*3/CYP2C9*3</i>                    | 3 (0.9)          |
| Alleles frequency (N = 664 alleles)         |                  |
| Allele                                      | Frequency; n (%) |
| <i>CYP2C9*1</i>                             | 527 (79.4)       |
| <i>CYP2C9*2</i>                             | 75 (11.3)        |
| <i>CYP2C9*3</i>                             | 62 (9.3)         |

**Table 2** Genotype and alleles frequencies observed for the *VKORC1* -1693 G>A polymorphism in our study

| Genotype distribution (N = 332 individuals) |                  |
|---|------------------|
| Genotype                                    | Frequency; n (%) |
| GG  | 107 (32.2)       |
| GA  | 170 (51.2)       |
| AA  | 55 (16.6)        |
| Alleles frequency (N = 664 alleles)         |                  |
| Allele                                      | Frequency; n (%) |
| G   | 384 (57.8)       |
| A   | 280 (42.2)       |

*CYP2C9* polymorphisms were consistent with the Hardy–Weinberg equilibrium (data not shown). The results regarding the distribution of *CYP2C9\*2* and *CYP2C9\*3* are shown in detail in Table 1. The absence of the *CYP2C9\*2* or *CYP2C9\*3* polymorphisms was considered as a wild-type allele and was denoted as '*CYP2C9\*1*'.

### ***VKORC1* -1639 G>A**

A total number of 170 individuals (51.2%) were heterozygous for the -1639 G>A polymorphism, whereas 55 individuals (16.6%) had a homozygous AA genotype. Thus, the A allele had a frequency of 42.2% in our group. The observed genotype frequencies for *VKORC1* -1639 G>A were consistent with the Hardy–Weinberg equilibrium (data not shown). The results regarding the distribution of the *VKORC1* -1639 G>A polymorphism are presented in detail in Table 2.

**Table 3** Allelic frequencies of *CYP2C9\*2* and *CYP2C9\*3* polymorphisms in Romanians compared with different populations

| Population               | Sample size | <i>CYP2C9*2</i> allele frequency (P-value*) | <i>CYP2C9*3</i> allele frequency (P-value*) | Reference     |
|--------------------------|-------------|---|---|---------------|
| Romanian                 | 332         | 11.3  | 9.3   | Present study |
| Faroese                  | 312         | 8.8 (NS)                                    | 5.3 (0.008)                                 | [8]           |
| French                   | 151         | 15 (0.01)                                   | 8 (NS)                                      | [9]           |
| Belgian                  | 121         | 10 (NS)                                     | 7.4 (NS)                                    | [10]          |
| Spanish                  | 157         | 14.3 (NS)                                   | 16.2 (<0.001)                               | [11]          |
| Italian                  | 360         | 12.5 (NS)                                   | 9.7 (NS)                                    | [12]          |
| Hungarian                | 535         | 12.5 (NS)                                   | 8.8 (NS)                                    | [13]          |
| Croatian                 | 200         | 16.5 (0.001)                                | 9.5 (NS)                                    | [14]          |
| Russian                  | 290         | 10.5 (NS)                                   | 6.7 (0.04)                                  | [15]          |
| Greek                    | 283         | 12.9 (NS)                                   | 8.13 (NS)                                   | [16]          |
| Turkish                  | 499         | 10.6 (NS)                                   | 10 (NS)                                     | [6]           |
| Chinese                  | 394         | 0.1 (<0.0001)                               | 3.6 (<0.0001)                               | [9]           |
| Japanese                 | 140         | 0 (<0.0001)                                 | 1.8 (<0.0001)                               | [17]          |
| Indian                   | 346         | 4 (<0.0001)                                 | 8 (NS)                                      | [18]          |
| Korean                   | 574         | 0 (<0.0001)                                 | 1.1 (<0.0001)                               | [3]           |
| Egyptian                 | 247         | 12 (NS)                                     | 6 (0.007)                                   | [19]          |
| Beninese                 | 111         | 0 (<0.0001)                                 | 0 (<0.0001)                                 | [10]          |
| Brazilian                | 331         | 8.6 (NS)                                    | 6.5 (0.003)                                 | [20]          |
| Bolivian                 | 778         | 4.8 (<0.0001)                               | 3 (<0.0001)                                 | [21]          |
| Canadian (Native Indian) | 153         | 3 (<0.0001)                                 | 6 (0.007)                                   | [22]          |
| Inuit                    | 151         | 0 (<0.0001)                                 | 0 (<0.0001)                                 | [22]          |

\*Fisher's exact test of Romanians *versus* other populations.

**Table 4** Allelic frequencies of the *VKORC1* -1639 G>A polymorphism in Romanians compared with other populations

| Population        | Sample size | Frequency of the A allele ( <i>P</i> -value*) | Reference     |
|-------------------|-------------|---|---------------|
| Romanian          | 332         | 42.2  | Present study |
| Hungarian         | 510         | 39 (NS)                                       | [23]          |
| Greek             | 98          | 48.5 (NS)                                     | [24]          |
| French            | 222         | 42 (NS)                                       | [5]           |
| Italian           | 147         | 39.8 (NS)                                     | [25]          |
| Lebanese          | 161         | 52.8 (NS)                                     | [26]          |
| Iranian           | 126         | 55.56 (NS)                                    | [27]          |
| Japanese          | 341         | 91.8 (<0.0001)                                | [28]          |
| Chinese           | 178         | 91.6 (<0.0001)                                | [29]          |
| Indian (Northern) | 102         | 14.22 (<0.0001)                               | [30]          |
| South African     | 993         | 4 (<0.0001)                                   | [31]          |

\*Fisher's exact test of Romanians *versus* other populations.

### ***CYP2C9*\*2, *CYP2C9*\*3 and *VKORC1* -1639 G>A in Romanians compared with other populations**

Table 3 shows the distribution of *CYP2C9*\*2 and *CYP2C9*\*3 across various populations and the differences in the allelic frequencies of these polymorphisms between these populations and the Romanians, as calculated by Fisher's exact test. Table 4 shows the distribution of the *VKORC1* -1639 G>A polymorphism across various populations and the differences in allelic frequencies (for the A allele) between Romanians and these populations, as calculated by Fisher's exact test.

## **Discussions**

*CYP2C9*\*2 and *CYP2C9*\*3, as demonstrated by our study, have a much higher frequency in Romanians than in Asian (Chinese, Japanese, Korean) or African populations (Ethiopians, Beninese), where these variants are very rare or sometimes absent. The two *CYP2C9* polymorphisms were also significantly enriched in Romanians compared with South Americans (Bolivians, Brazilians), which have intermediate frequencies between Asians and Europeans. However, the allelic frequencies of *CYP2C9*\*2 and *CYP2C9*\*3 observed in our group are similar to those observed in most of the Caucasian populations living in Europe. In our group, 109 individuals (32.8%) were *CYP2C9*\*2 or *CYP2C9*\*3 heterozygotes, predicting a *CYP2C9* intermediate enzymatic activity, whereas 14 individuals (4.2%) are predicted to have the lowest *CYP2C9* enzymatic activity (those with *CYP2C9*\*2 homozygous, *CYP2C9*\*3 homozygous and *CYP2C9*\*2/*CYP2C9*\*3 compound heterozygous genotype). Assuming that *CYP2C9*\*2 and *CYP2C9*\*3 are homogeneously distributed in all

Romanians and that other *CYP2C9* defective alleles are rare in our population, we expect approximately 7,770,000 individuals harbouring at least one *CYP2C9*\*2 or *CYP2C9*\*3 allele, taking into account the population of Romania with approximately 21,000,000 inhabitants. Moreover, approximately 882,000 Romanians are expected to have a *CYP2C9*\*2 homozygous, *CYP2C9*\*3 homozygous or *CYP2C9*\*2/*CYP2C9*\*3 compound heterozygous genotype. These individuals would have the lowest *CYP2C9* enzymatic activity, with respect to the *CYP2C9*\*2 and *CYP2C9*\*3 alleles. This frequency becomes important for the *CYP2C9* substrates prescribed in Romania, but especially for the oral anticoagulants, in which the *CYP2C9* status has major implications.

In Asians (*e.g.* Chinese, Japanese), the *VKORC1* -1639 G>A polymorphism is extremely prevalent, with allelic frequencies for the A allele usually greater than 90%. In general, the A allele has an increasing frequency from Western Europe towards Eastern Asia, a finding that explains, at least in part, the inter-ethnic variations in the dosage requirements of the oral anticoagulants, with the Asians requiring lower doses for efficient anticoagulation. However, we found the frequency of the A allele in the Romanian group to be 42.2%, which is in good concordance with most of the Caucasian populations living in Europe, where the frequency of this allele is approximately 40%. We observed the GA and AA genotypes in 225 individuals (67.8%), which represent roughly two thirds of the group analysed. By extrapolation to the entire Romanian population, we may expect an approximate number of 14,000,000 inhabitants who would require lower doses of the oral anticoagulants. In our group, the AA homozygous genotype was observed in 55 individuals (16.6%), which represents roughly one sixth of the group analysed. Thus, we may expect approximately 3,500,000 Romanian inhabitants who would require the lowest dose of oral anticoagulants, based on the *VKORC1* -1639 G>A status.

All the participants to this study were Romanians. The projections we made regarding the distribution of *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms assume a relatively homogeneous population. We made this assumption because Romania has indeed a relatively homogeneous population, around 90% of the inhabitants being Romanians. However, we admit that in certain sub-populations living in our country, we might have a different distribution of these polymorphisms, given that in general these sub-populations were not admixed with the Romanians over the centuries. Obviously, this would have important consequences regarding the oral anticoagulants metabolism in those sub-populations.

The last years have proven that the oral anticoagulants have a more complex metabolism. Along the *CYP2C9* and *VKORC1*, new genes, such as *CYP4F2*, *GGCX* and *CALU* (the latter especially in African Americans) emerged as modifiers of the dose requirements [32]. It would be helpful to evaluate the frequencies of the *CYP4F2* rs2108622 polymorphism, which contributes to 1–7% of mean weekly warfarin dose variance and *GGCX* rs11676382 polymorphism, which contributes to a 6.1% reduction in warfarin dose requirement per G allele, to obtain a more complete pharmacogenomic profile of the oral anticoagulants in our country.

## Conclusions

This report is the first on the distribution of the *CYP2C9* and *VKORC1* -1639 G>A polymorphisms in a Romanian population. The distribution that we found for these polymorphisms is in good agreement with those for other European populations.

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The genetic variation at the *CYP2C9* and *VKORC1* loci accounts for most of the inter-individual variability in the dosage requirements of oral anticoagulants. We expect a large number of Romanians harbouring at least one *CYP2C9* detrimental variant or at least one A allele at the *VKORC1* -1639 G>A position. The oral anticoagulants (especially acenocoumarol) are widely prescribed in our country; thus, a large number of these patients are at risk of over-anticoagulation, at least in the induction phase of the treatment. Genotyping for the *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* -1639 G>A polymorphisms should be performed whenever possible, prior to the initialization of the therapy, to identify the individuals at risk of over-anticoagulation. Moreover, new genetic polymorphisms such as *CYP4F2* rs2108622 and *GGCX* rs11676382, as well as other *CYP2C9* alleles, should be evaluated on the Romanian population to have a more encompassing profile of the oral anticoagulants pharmacogenomics.

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A.P.T. performed the genetic analyses, analysed the data and wrote the manuscript; S.C. collected the samples and analysed the data; A.D.B. designed the research and revised the manuscript and D.F.M. revised the manuscript.

## Conflicts of interest

The authors confirm that there are no conflicts of interest.

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