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**Basic Science**

## Distribution of *CYP2C9* and *VKORC1* Gene Polymorphisms in Healthy Macedonian Male Population

Krume Jakjovski<sup>1</sup>, Nikola Labachevski<sup>1</sup>, Aleksandar Petlichkovski<sup>2</sup>, Aleksandar Senev<sup>2</sup>, Jasmina Trojancanec<sup>1</sup>, Emilija Atanasovska<sup>1</sup>, Elena Kostova<sup>1</sup>, Mirko Spiroski<sup>2</sup>

<sup>1</sup>*Institute of Preclinical and Clinical Pharmacology with Toxicology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Republic of Macedonia;* <sup>2</sup>*Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Republic of Macedonia*

### Abstract

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**Key words:** gene polymorphisms; alleles; *CYP2C9*; *VKORC1*; genotyping; Macedonian population.

**Correspondence:** Dr. Krume Jakjovski. Institute of Preclinical and Clinical Pharmacology with toxicology, Faculty of Medicine, 50. Divizija No 6, Skopje 1109, Republic of Macedonia. E-Mail: jakjovski@gmail.com

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**Competing Interests:** The authors have declared that no competing interests exist.

**Background:** Distribution of *CYP2C9* and *VKORC1* gene polymorphisms may vary significantly among different ethnic groups, and eventually influence the variation in drug metabolism or even failure.

**Objective:** The aim of this study was to evaluate the prevalence of *CYP2C9* and *VKORC1* alleles in the healthy population of Republic of Macedonia compared to the global geographic data reported from different ethnic populations. Also, to genotype *CYP2C9* and *VKORC1* genes and eventually to divide individuals in poor, extensive, or intermediate metabolizer.

**Material and Methods:** Blood samples were collected after signing written consent, DNA was isolated from peripheral blood, and *CYP2C9* and *VKORC1* genes were typed (n=124). Genotyping was performed by commercially available kits (GeneID GmbH, Strassberg, Germany, AID Diagnostica), based on the method of polymerase chain reaction with a subsequent hybridization. The population genetics analysis package, PyPop ver. 0.6.0, was used for analysis of the data.

**Results:** The frequency of alleles varies from 0.931 for *CYP2C9*\*3 to 0.109 for *CYP2C9*\*2 indicating common "wild type" allele in those genes. The frequency ranges spanned ~50% for each allele of *VKORC1* gene, indicating no common "wild type" allele in this gene. Test of neutrality showed significant negative value for *VKORC1* polymorphism that indicates balancing selection operating on the alleles at that locus. All polymorphisms of *CYP2C9*\*2, *CYP2C9*\*3 and *VKORC1* showed a good fit with Hardy-Weinberg expectations.

**Conclusion:** The results of polymorphic alleles of *CYP2C9* and *VKORC1* genes in Macedonian population can be used for the variation in drug metabolism studies as well for adapting dosage regimes for oral anticoagulant therapies.

### Introduction

The cytochrom P450 (CYP) family of heme monooxygenases comprise the most important group of Phase I enzymes. These enzymes oxidize a wide range of endogenous, as well as exogenous compounds. The cytochrome P450 gene family contains 60-100 different genes, of which only a small group is involved in drug and chemical transformations. The most important P450 isoenzyme is *CYP3A4* (50% of the P450 metabolism) followed by *CYP2D6* (20%), *CYP2C9* and *CYP2C19* (together 15%). The remaining is carried out by *CYP2E1*, *CYP2A6* and *CYP1A2* [1].

The genes for *CYP2D6*, *CYP2C9*, *CYP2C19* and *CYP2A6* are functionally polymorphic. Therefore,

approximately 40% of human P450 dependent drug metabolism is carried out by polymorphic enzymes (for a list of all currently known cytochrome P450 gene alleles go to <http://www.cypalleles.ki.se/>) [2].

There are 3 *CYP2C9* alleles - *CYP2C9*\*1 allele is wild-type, but *CYP2C9*\*2 and *CYP2C9*\*3 are mutated alleles. In both mutated alleles, a point mutation leads to a substitution of an amino acid in the protein. In *CYP2C9*\*2 an arginine at position 144 is converted to cysteine (R144C), in *CYP2C9*\*3 an isoleucine at position 359 is converted to leucine (I359) Cytochrom P450 *CYP2C9* catalyse the metabolism of important drugs such as phenytoin, tolbutamide, losartan, warfarin, and several NSAIDs [3]. Interestingly, significant variation in distribution of polymorphic variants of the CYP450 genes is

documented among different populations worldwide [4-25]. The vitamin K epoxide reductase (VKOR) is a membrane protein in the endoplasmic reticulum, which 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 is pharmacodynamic target of oral anticoagulants and is encoded by the VKORC1 gene.

Macedonia is located in the Central Balkans, bordering Bulgaria, Greece, Albania, Serbia and Kosovo province, covering an area of 25,710 kms. According to the 2002 census, the country's population was 2,022,577. Data on the declared ethnic affiliation from the 2002 census reported that 64.1% of the population identify themselves as Macedonian, 25.17% as Albanian, 3.95% as Turks, 2.66% as Roma, 1.78% as Serbs, 0.84% as Bosniacs, 0.48% as Vlachs and 1.04% others [26]. The country seceded peacefully from Yugoslavia after an independence referendum, held in September 1991.

This is the first study of the diversity of polymorphisms of *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* in Macedonian populations. The aim of this study was to determine the frequencies of *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* alleles and genotypes and to compare them with populations for which similar data are available.

## Material and Methods

### Population

Unrelated healthy male volunteers of Macedonian origin (n=124), between the age of 18-55 years with a mean age of 36.43 ( $\pm$  3.47) years, which are subjects of bioequivalence studies, were included in the study. All individuals are of Macedonian origin, nationality, and residents of the geographical areas of different regions of the Republic of Macedonia. From each individual was obtained signed informed consent prior to his or her enrolment. The Ethical Committee of the Faculty of Medicine in Skopje has approved the study. After admission to the study, blood samples were collected from each subject (5 ml) into an EDTA tube, and the genomic DNA was isolated from peripheral blood leukocytes by the phenol-chloroform extraction method [27]. Samples were stored in Anthropology field of the Macedonian Human DNA Bank (hDNAMKD) [28].

### Typing methods

*CYP2C9\*2* (rs1799853), *CYP2C9\*3* (rs1057910), and *VKORC1 C1173T* (rs 9934438) polymorphisms genotyping was performed by commercially available kits from GeneID GmbH, Strassberg, Germany. It is based on the method of

polymerase chain reaction (PCR) with a subsequent hybridization. A multiplex PCR is first carried out with DNA isolated from whole blood. In this reaction, two fragments of the *CYP2C9* gene and one fragment of the *VKORC1* gene are amplified with specific biotin labelled primers. The amplified gene fragments are then characterized in a hybridization reaction with sequence-specific oligonucleotide probes (SSOP), which are immobilized on nitrocellulose (reverse hybridization). Each nitrocellulose strip has gene probes for the wild-type and mutated sequences of the tested gene fragments, as well as control zones.

### Statistical methods

The population genetics analytics package, PyPop ver. 0.6.0 [29], was used for analysis of the gene data. Allele frequencies and expected Hardy Weinberg proportions (HWP) [30] for each single nucleotide polymorphisms (SNP) were determined. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop. Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotypes were significantly different from expected frequencies by the Chi-square test. The Ewens-Watterson homozygosity test of neutrality with Slatkin's p-values [31] was used to indicate any deviations from the hypothesis of neutral selection for each locus. P-value less than 0.05 was taken as significant.

## Results

### *CYP2C9* and *VKORC1* Alleles

Frequencies of polymorphic alleles, test of neutrality with  $F_{nd}$  statistic [Ewens-Watterson test of neutrality (EWN)], and Slatkin's Exact p-value (SEPV) with p of F statistics in macedonian population are shown on the Table 1. The frequency of alleles varies from 0.931 for *CYP2C9\*3* to 0.109 for *CYP2C9\*2*, indicating common "wild-type" allele in these genes. The frequency ranges spanned ~50% for each allele of *VKORC1* gene, indicating no common "wild type" allele in this gene (Table 1).

**Table 1: Frequencies of polymorphic alleles, test of neutrality with  $F_{nd}$  statistic (Ewens-Watterson test of neutrality (EWN)), and Slatkin's Exact P. Value (SEPV) with p of F statistics in Macedonian population.**

Polymorphism	Alleles			Test of neutrality	
	Allele	Number	Frequency	$F_{nd}$	p of F
<i>CYP2C9*2</i>	*1	221	0.891	-0.186	0.346
	*2	27	0.109		
<i>CYP2C9*3</i>	*1	231	0.931	0.210	0.430
	*3	17	0.069		
<i>VKORC1</i>	C	134	0.540	-1.995	0.027*
	T	114	0.460		

Statistically significant  $F_{nd}<0$  indicates balancing selection; significant  $F_{nd}>0$  indicates directional selection; \*statistically significant.

Test of neutrality showed negative values for  $F_{nd}$  statistics (Ewens-Watterson test of neutrality) for *CYP2C9\*2* and *VKORC1* polymorphism (-0.186 and -1.995, respectively), which indicates balancing selection operating on the alleles at that locus, but significantly different from 0 only for the *VKORC1* gene [Slatkin's exact P value (SEPV) 0.346 and 0.027, respectively].  $F_{nd}$  was positive and none significantly differs from 0 for *CYP2C9\*3* ( $F_{nd}=0.210$  and  $p$  of  $F=0.430$ ) (Table 1).

*CYP2C9 and VKORC1 genotypes*

Twenty-three individuals (18.6%) were heterozygous for *CYP2C9\*2*, whereas 17 individuals (13.7%) were heterozygous for *CYP2C9\*3*. There were two individuals (1.6%) which are homozygous for *CYP2C9\*2*, but no individual was homozygous for *CYP2C9\*3*. In addition, there were no individuals declared as heterozygotes (*\*2/\*3*). Thus, only 2 individuals can be predicted as subjects to have the lowest *CYP2C9* enzymatic activity, with respect to *\*2* and *\*3*; so they may be declared as PM (poor metabolizers).

**Table 2: Observed vs expected CYP2C9\*2, CYP2C9\*3 and VKORC1 genotypes, Hardy Weinberg proportions (HWP), and Guo Thompson Hardy Weinberg Output (GTHWO) in macedonian population.**

Polymorphism	Genotype	Observed number (n)	Observed frequency (%)	Expected number (%)	p-value	HWP p-value	GTHWO p-value
<i>CYP2C9*2</i>	<i>*1/*1</i>	99	79.8	98.5	0.829	0.824	0.634
	<i>*1/*2</i>	23	18.6	24.1	0.957		
	<i>*2/*2</i>	2	1.6	1.5	&		
<i>CYP2C9*3</i>	<i>*1/*1</i>	107	86.3	107.6	0.995	0.765	1.00
	<i>*1/*3</i>	17	13.7	15.8	0.769		
	<i>*3/*3</i>	0	0	0.6	&		
<i>VKORC1</i>	<i>C/C</i>	37	29.8	36.2	0.894	0.772	0.858
	<i>C/T</i>	60	48.4	61.6	0.838		
	<i>T/T</i>	27	21.8	26.2	0.876		

& Can not be calculated because expected <5,  $\chi^2$  test.

Overall, the *CYP2C9\*2* allele had a frequency of 10.1%, whereas the *CYP2C9\*3* allele had a frequency of 6.8%. The observed genotype frequencies for both polymorphisms for *CYP2C9* were consistent with Hardy Weinberg equilibrium (Table 2).

Sixty individuals (48.4%) were heterozygous for *VKORC1* polymorphism, whereas 27 individuals (21.8%) had a homozygous genotype. The observed genotype frequencies for *VKORC1* were consistent with Hardy Weinberg equilibrium (Table 2).

Observed versus expected genotypes for each SNP, Hardy-Weinberg proportion (HWP), and Guo and Thomson Hardy Weinberg Output (GTHWO) is given in the Table 3. All polymorphisms of *CYP2C9\*2*, *CYP2C9\*3* and *VKORC1* showed a good fit with HWP expectations (HWP p-value=0.824, 0.765 and 0.772, respectively). In two instances,  $\chi^2$  test cannot be calculated because expected frequency was smaller than 5 (*CYP2C9\*2 \*2/\*2* and *CYP2C9\*3 \*3/\*3*).

**Table 3: Comparison of CYP2C9 allelic frequencies reported from different ethnic groups and different geographic regions.**

Population	n	CYP2C9 variant allele			Reference
		Frequency, *1	Frequency, *2	Frequency, *3	
<b>Caucasians</b>					
Republic of Macedonia	124	0.831 (referent)	0.101 (referent)	0.068 (referent)	This study
Spain	157	0.70(NS)	0.14(NS)	<b>0.16 (0.001)</b>	[4]
Italy	360	0.77(NS)	0.12(NS)	0.09(NS)	[5]
Slovenia	129	0.82(NS)	0.12(NS)	0.06(NS)	[6]
Croatia	200	0.74(NS)	0.16(NS)	0.09(NS)	[7]
Greece	283	0.79(NS)	0.13(NS)	0.08(NS)	[8]
France	151	0.77(NS)	0.15(NS)	0.08(NS)	[9]
Germany	266	0.81(NS)	0.11(NS)	0.08(NS)	[10]
Belgium	121	0.82(NS)	0.10(NS)	0.07(NS)	[11]
Sweden	430	0.82(NS)	0.17(NS)	0.07(NS)	[12]
Danmark	276	0.83(NS)	0.12(NS)	0.05(NS)	[13]
Britain	100	0.84(NS)	0.13(NS)	0.08(NS)	[14]
Russia	290	0.83(NS)	0.10(NS)	0.07(NS)	[15]
Hungary	332	0.86(NS)	0.11(NS)	0.09(NS)	[16]
America	100	0.85(NS)	0.08(NS)	0.06(NS)	[17]
Canada	114	0.81(NS)	<b>0.03(0.007)</b>	0.06(NS)	[18]
Turkey	499	0.79(NS)	0.11(NS)	0.10(NS)	[10]
<b>Hispanic</b>					
Mexican	169	0.86(NS)	<b>0.08(0.029)</b>	0.06(NS)	[19]
America Brazil	331	0.86(NS)	<b>0.07(0.017)</b>	0.07(NS)	[20]
<b>African</b>					
Africa	993	0.99(NS)	<b>0.02(0.001)</b>	<b>0.01(0.025)</b>	[21]
America	100	0.98(NS)	<b>0.01(1*10<sup>-7</sup>)</b>	<b>0.005(0.0002)</b>	[17]
Egypt	247	0.82(NS)	0.12(NS)	0.06(NS)	[22]
<b>Asian</b>					
Vietnam	157	0.98 (NS)	0 (<0.0001)	<b>0.02(0.0017)</b>	[23]
Japan	218	<b>0.98(0.037)</b>	0 (<0.0001)	<b>0.021(0.0008)</b>	[24]
Korea	574	<b>0.99(0.011)</b>	0 (<0.0001)	0.011 (<0.0001)	[24]
China	98	0.97(NS)	0 (<0.0001)	<b>0.026(0.031)</b>	[24]
South India	346	0.88(NS)	0.04 ( <b>3*10<sup>-7</sup></b> )	0.08(NS)	[25]

Table 3 shows the distribution of frequencies of the *CYP2C9* and *VKORC1* variant alleles in the healthy population of Republic of Macedonia compared to data reported from various ethnic groups and global geographic regions.

Allelic frequencies of rs9934438 (*1173C>T*) polymorphism in *VKORC1* in different countries/regions are presented in Table 4. The lowest frequency of the *VKORC1* CC genotype was reported in Chinese populations (1%, 1.11% and 2.9% respectively) [33, 35, 39]. The highest frequencies for *VKORC1* CC genotype were reported in Netherlands, Italy and USA (37.2%, 36.8%, and 36.1% respectively). The frequency of *VKORC1* CC genotype in Macedonians was similar to Caucasians (29.8%) (Table 4).

**Table 4: Allelic frequencies of rs9934438 (1173C>T) polymorphism in VKORC1 in different countries/regions.**

Reference SNP	Genotypes			Country/region	Reference
rs9934438 (1173C>T)	CC (%)	CT (%)	TT (%)		
	29.8	48.4	21.8	Republic of Macedonia	This paper [34]
	36.8	46.9	16.3	Italy	[32]
	36.1	50.7	12.7	USA	[41]
	34	49	17	France	[35]
	1	14	85	China	[33]
	1.11	14.7	84.1	China	[39]
	2.9	15.7	81.4	China	[36]
	6	25	69	Hong Kong	[37]
	11	24	65	Egypt	[38]
	37.2	48	14.8	Netherlands	

Homozygous genotypes for *VKORC1* TT were dominant in the yellow populations (85%, 69% and 65% in China, Hong Kong and Egypt, respectively). Dominant heterozygous *VKORC1* CT genotypes were reported in all Caucasian populations

(Republic of Macedonia, Italy, USA, France and Netherlands) with frequencies between 46.9% and 50.7 (Table 4).

## Discussion

In this study we present the distribution of the common alleles of *CYP2C9* that has been found to vary across populations and different ethnic groups. The extent of variation is directly related to the genetic distance between populations and the allele being examined [40].

Results which are obtained from our study, showed that the allelic frequencies of *CYP2C9*\*2 (10.1%) and *CYP2C9*\*3 (6.8%) in tested population were similar to the general frequencies reported for the Caucasian population throughout Europe [41].

Compared to the frequency of *CYP2C9*\*3 found in the Spanish ethnic group (16.2%) [4] a significant interethnic difference was noticed.

The *CYP2C9*\*2 allele is not found in the Asian population, prevalent in approximately 15% in Caucasians and variable in populations with African ancestry (1-3.6% African Americans, 4.3% Ethiopians). *CYP2C9*\*3 is estimated to 0.5-2.3% in Africans and 1.1-6.8% in Asians, but it is more common in Caucasians (3.3-17%) [41-44].

Several meta-analyses were published about the role of *VKORC1* polymorphisms and different clinical conditions. To conduct a systemic review and meta-analysis to investigate the relationship between mean daily warfarin dose (MDWD) and *VKORC1* single nucleotide polymorphisms (SNPs), a total of 19 studies were included in the meta-analysis. The frequencies of 1173TT and -1639 AA in Asian patients were higher than those in Caucasian and African populations. Sensitive analyses demonstrated that the impacts of gene polymorphism on warfarin dosage requirement were significantly different between Caucasian and Asian population, and the results of meta-analyses were stable and reliable [45]. Navigating the literature to determine how genotype will influence warfarin response in a particular patient is difficult, due to significant variation in patient ethnicity, outcomes investigated, study design, and methodological rigor. A paper was published where comprehensive search strategy was applied and 117 studies included [46]. Primary outcomes were stable dose, time to stable dose and bleeding events. Assessing study quality highlighted significant variability in methodological rigor. Notably, there was significant evidence of selective reporting, of outcomes and analysis approaches [46].

## Conclusion

This study is the firsts on the distribution of the

*CYP2C9* and *VKORC1* polymorphism in Macedonian population. This study shows that *CYP2C9* and *VKORC1* are polymorphic in the Macedonian population. Our data show that the distribution of *CYP2C9* variant alleles frequencies are similar to the most of those reported for Caucasians of European descendant, but differ from those of North America Caucasians. Results from this study suggest that the genetically determined capacity of *CYP2C9* has to be taken into account in process of designing, conducting and evaluation of pharmacokinetics for investigational drugs [44].

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