sciendo

10.2478/AMB-2025-00057

ORIGINAL ARTICLE

POLYMORPHIC VARIANTS IN ACENOCOUMAROL-RELATED GENES *CYP2C9* AND *VKORC1* AND THEIR FREQUENCIES IN BULGARIAN POPULATION

R. Tzveova[1], A. Dimitrova-Karamfilova[2], S. Vandeva[3], P. Atanasov[4], D. Pendicheva-Duhlenska[5], G. Nachev[6], V. Mitev[7], R. Kaneva[7]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences – Sofia, Bulgaria
[2]Department of Clinical Laboratory, XXV Diagnostic Consultative Center – Sofia, Bulgaria
[3]Clinical Center of Endocrinology and Gerontology, Medical University – Sofia, Bulgaria
[4]National Sports Academy, Sofia, Bulgaria
[5]Department of Pharmacology, Faculty of Pharmacy, Medical University – Pleven, Bulgaria
[6]Department of Cardiovascular Surgery, University National Multi-profile Hospital for Active Treatment "Sv. Ekaterina" – Sofia, Bulgaria
[7]Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical Faculty, Medical University – Sofia, Bulgaria

Abstract. Introduction. *Acenocoumarol is an indirect anticoagulant, a 4-hydroxycoumarin derivative, which is used in the treatment of various thromboembolic diseases. Polymorphic variants in the CYP2C9 and VKORC1 genes significantly contribute to the optimal maintenance acenocoumarol dose requirements in different populations.* ***Purpose.*** *The purpose of the study was to establish the allele and genotype frequencies of the following polymorphic variants: CYP2C9*2; CYP2C9*3; VKORC1*2A; VKORC1*2B; VKORC1*3 and VKORC1*4 in Bulgarian participants and to compare them to these in other European populations.* ***Materials and Methods.*** *Our study included 155 unrelated controls from different regions of Bulgaria. Genomic DNAs were extracted from peripheral venous blood. The analysis of selected polymorphisms was performed by High Resolution Melting Analysis. The allele frequencies of various genotypes were calculated by Hardy–Weinberg Equilibrium (HWE).* ***Results.*** *The obtained frequencies of polymorphisms studied in this study are presented below: 1. CYP2C9*2 – CC (85.71%), CT (13.47%), TT (0.82%), C (92.00%), T (8.00%), HWE χ^2=2.58, 2. CYP2C9*3 – CC (0.04%), CT (16.94%), TT (82.66%), C (8.87%), T (91.13%), HWE χ^2=0.26, 3. VKORC1*2A – GG (32.47%), GA (48.70%), AA (18.83%), G (56.82%), A (43.18%), HWE χ^2=0.01, 4. VKORC1*2A – GG (32.47%), GA (48.70%), AA (18.83%), G (56.82%), A (43.18%), HWE χ^2=0.01, 5. VKORC1*2B – GG (11.43%), GT (44.29%), TT (44.29%), G (33.57%), T (66.43%), HWE χ^2=0.01, 6. VKORC1*3 – GG (41.04%), GA (48.23%), AA (9.93%), G (65.96%), A (34.04%), HWE χ^2=0.77, 7. VKORC1*4 – CC (64.93%), CT (32.09%), TT (2.99%), C (80.97%), T (19.03%), HWE χ^2=1.51.* ***Conclusion.*** *The established genotypic and allelic frequencies of the studied polymorphisms in CYP2C9 and VKORC1 were according to the Hardy–Weinberg equilibrium and correspond to these in other European populations.*

Key words: *CYP2C9, VKORC1, acenocoumarol, frequencies, Bulgarians*

Corresponding author: *Reni Tzveova, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 25, Sofia, Bulgaria, email: renitzveova@abv.bg*

ORCID: *0000-0001-5045-8165*

Received: *27 January 2025;* ***Revised/Accepted:*** *17 March 2025*

INTRODUCTION

Thromboembolism is a major cause of morbidity and mortality because of myocardial infarction and ischemic stroke. The main drugs used to both prevent and treat patients with thrombosis are the indirect anticoagulants such as acenocoumarol.

Acenocoumarol, a vitamin K antagonist used for anticoagulation therapy, exhibits significant interindividual variability in dosing requirements, largely influenced by genetic factors. The primary genes implicated in this variability are *VKORC1* and *CYP2C9*, with polymorphisms in these genes affecting the drug's pharmacokinetics and pharmacodynamics. Studies across various populations, including Serbian, Chilean, Spanish, Tunisian, Greek, Mexican, and Vietnamese cohorts, have consistently demonstrated the impact of *VKORC1* and *CYP2C9* variants on acenocoumarol dosing. For instance, *VKORC1* variants are associated with both hypersensitivity and resistance to acenocoumarol, necessitating dose adjustments to achieve therapeutic efficacy [1].

In a Serbian study, a mathematical model incorporating these genetic factors predicted individual dosing with significant accuracy, highlighting the potential for population-specific dosing algorithms [1].

Similarly, a Chilean study developed an algorithm that explained nearly 50% of the variability in therapeutic dosing, emphasizing the role of *VKORC1* and *CYP2C9* polymorphisms alongside clinical factors like age and BMI [2].

In a randomized clinical trial in Spain, a pharmacogenetic dosing algorithm significantly improved the proportion of patients achieving target INR levels compared to standard care, underscoring the clinical utility of such approaches [3]. Furthermore, a genome-wide association study in Spain identified additional polymorphisms associated with acenocoumarol maintenance dose, stroke recurrence, and intracranial hemorrhage, suggesting broader implications for genetic testing in anticoagulant therapy [4].

The inclusion of other genetic factors, such as *CYP4F2*, has been explored in Greek and Mexican populations, indicating potential improvements in dosing algorithms when considering a wider array of genetic markers [5, 6].

Overall, the integration of pharmacogenetic data into clinical practice could enhance the precision of acenocoumarol dosing, reduce adverse events, and improve therapeutic outcomes across diverse patient populations [3, 4].

At the present time, the monitoring of the response to acenocoumarol therapy in Bulgaria is currently based on the measurement of prothrombin time monthly or every few months, which can limit patient compliance. It is necessary to establish and implement methodologies that enable the optimization of acenocoumarol therapy. These methods should be reliable and insure that the individual response to treatment for each patient be accurately determined and the results obtained do not change over time. It is also essential to consider the influence of genetic factors on acenocoumarol maintenance dose requirement and response to treatment.

The molecular changes in metabolizing enzymes may lead to the slower or more rapid metabolization of the drug of concern. This may cause an overabsorption or insufficient amounts of the drug at the receptor site, regardless of the administered standard drug dose. The pharmacokinetic effects are the result of inter-individual differences in absorption, distribution, metabolism (activation of prodrug, inactivation of biologically active molecules with biological activity) or excretion of the medication. The pharmacodynamic effects can lead to interindividual differences in drug effects and drug responses regardless the presence of appropriate concentrations of an active compound at the target site of application.

MATERIALS AND METHODS

Our study included 155 unrelated controls from different regions of Bulgaria selected from DNA biobank of Molecular Medicine Center, Medical University – Sofia. Genomic DNA was isolated from peripheral venous blood samples using Chemagic Magnetic Separation Module I (PerkinElmer) according to the manufacturer's protocol. The analysis of selected polymorphisms *CYP2C9**2; *CYP2C9**3; *VKORC1**2A; *VKORC1**2B; *VKORC1**3 and *VKORC1**4 was performed by High Resolution Melting Analysis (Rotor Gene, Qiagen). The allele frequencies of various genotypes were calculated by Hardy$Weinberg Equilibrium. P-value of ≤ 0.05 was considered statistically significant.

The subject of the investigation was explained to each patient, and each patient signed a voluntary informed consent to participate in the study. All participants were over 18 years of age.

The baseline characteristics of the population control group are summarized in Table 1.

Table 1. Baseline characteristics of population control group

Indicator	Value±SD/number (%)
Age (in years)	36.08±12.99
Sex (male)	75 (48.38)
BMI (kg/m²)	25.66±4.91
Total cholesterol (mmol/l)	4.99±0.94
Triglicerides (mmol/l)	0.93±0.56
LDL (mmol/l)	3.18±0.88
HDL (mmol/l)	1.62±0.40

OPTIMISATION AND VALIDATION OF DNA METHODS FOR ANALYSIS OF PHARMACOGENETIC POLYMORPHIC VARIANTS IN *CYP2C9 AND VKORC1 GENES*

The genotyping of polymorphic variants in the *CYP2C9* gene (*CYP2C9**2, 430C>T, rs1799853; and *CYP2C9**3, 1075A>C, rs1057910) and *VKORC1* (*VKORC1**2A, rs9923231, g. 3588 G>A; rs9934438, g.6399 C>T; *VKORC1**2B, rs2884737, g.5723T>G; *VKORC1**3, rs7294, g.8956 G>A; *VKORC1**4, rs17708472, g.5924C>T) were performed by high-resolution melting technology of double-stranded DNA structure under constant increase of temperature at regular time intervals (High Resolution Melting Analysis, HRMA). Each genotype was differentiated on the basis of the melting profile of the corresponding amplicons harboring the polymorphic variant of interest (Fig. 1). A number of samples of each genotype were confirmed by direct Sanger sequencing (ABI 3130xl Sequence Genetic Analyzer (Applied Biosystems) for validation of the method.

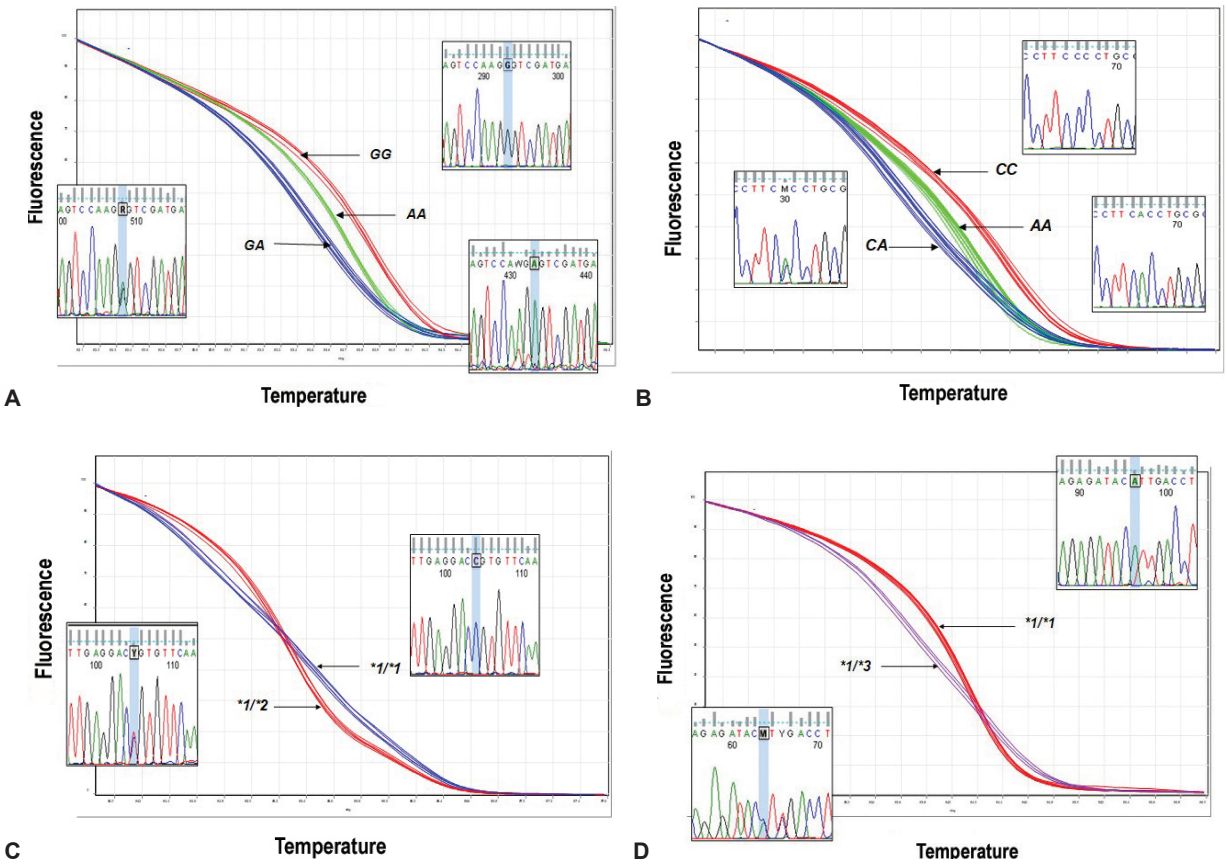
Optimization of the PCR reaction conditions ensured that a highly specific amplification product was obtained. The influence of the main reaction parameters (hybridization temperature, synthesis time, DNA concentration, MgCl2 concentration, primer concentration) on the sensitivity and reproducibility of the assay was investigated. The primer pairs and optimal conditions of ampliphication were selected for all the reactions (Table 2-3).

Table 2. Sequences of amplification primers for the regions containing the target polymorphisms in the *CYP2C9* and *VKORC1* genes

Polymorphic variant	Primers	Length of fragment (b.p.)	T of hybridization (0C)
CYP2C9*2 (430C>T) rs1799853	F: 5‘ – CCT GGG ATC TCC CTC CTA GT-3‘ R: 5’ – TTC CAA GAA TGT CAG TAG AGA AG-3’	257	60
CYP2C9*3 (1075A>C) rs1057910	F: 5’- TTG AAC GTG TGA TTG GCA GA-3’ R: 5’ – TCG AAA ACA TGG AGT TGC AG -3’	210	60
VKORC1 -1639G>A rs9923231	F: 5’- AGT TTG GAC TAC AGG TGC CTG-3’ R: 5’ – GCC AGC AGG AGA GGG AAA TA -3’	290	60
VKORC1 1173C>T rs9934438	F: 5’- GGG TGG AAC CAG GTT AGG AC-3’ R: 5’ – AAA GCA GGG CCT ACG GAG T -3’	234	58
VKORC1 (3730G>A) rs7294	F: 5’- CTG GGC AAT GGA AAG AGC-3’ R: 5’ – CAG GGC AAG GCT AAG AGG -3’	263	59
VKORC1 (497T>G) rs2884737	F: 5’- CAT CAC GCA CAG CCA GAC-3’ R: 5’ – CAA GGG ACC AGA CAG TGC T -3’	208	65
VKORC1 (698C>T) rs17708472	F: 5’- AGG CTG GTC AAC TCC TGA-3’ R: 5’ – GTC TGG CTG TGC GTG ATG -3’	271	66

Table 3. Optimization of conditions for amplification of selected polymorphic variants in *CYP2C9* and *VKORC1* genes

Polymorphic variant	ID	Chr: Position	Position	Replacement	Protein replacement	Type of change	T of hybridization (°C)	Time of amplification (sec.)
CYP2C9*2	rs1799853	10:94942290	Exon 3	C>T	Arg144Cys	missense	60	15:15:20
CYP2C9*3	rs1057910	10:94981296	Exon 7	A>C	Iso359Leu	missense	60	15:15:20
VKORC1*2A	rs9923231	16:31096368	5’UTR/ promotor	G>A	-	-	60	15:15:20
VKORC1*2A	rs9934438	16:31093557	intron 1	C>T	-	-	58	15:15:20
VKORC1*2B	rs2884737	16:31094233	intron 1	A>C	-	-	65	15:15:20
VKORC1*3	rs7294	16:31091000	3’UTR	C>T	-	-	59	15:15:20
VKORC1*4	rs17708472	16:31105353	intron 1	G>A	-	-	66	15:15:20



A: *VKORC1*2A* (*VKORC1* g.-1639G>A, rs9923231); **B:** *CYP2C9*2* (g.8633C>T, rs1799853); **C:** *CYP2C9**3 (g.16052G>A, rs1057310); **D:** *VKORC1**2B (g.5723T>G, rs2884737)

Fig. 1. Normalized melting graphs of selected polymorphic variants

DISTRIBUTIONS OF THE STUDIED POLYMORPHIC VARIANTS IN *CYP2C9 AND VKORC1 GENES IN THE* BULGARIAN POPULATION

The genotype and allele frequencies of the studied polymorphic variants in the *CYP2C9* and *VKORC1* genes in the group of healthy individuals (population controls) did not show any deviation from those expected according to the Hardy–Weinberg law (Table 4-5).

The next several tables present comparisons of allele and genotype frequencies in Bulgarians with those observed in other population groups (Table 6-11). The frequency data are obtained from the Ensembl database and are the result of work during the first phase of the 1000 genomes project.

Table 4. Allele and genotype frequencies of the studied polymorphic variants in the *CYP2C9* gene in a group of population controls of Bulgarian origin

Total	**n=144**		**n=148**	
Variant	*CYP2C9*2* rs1799853 g.8633C>T		*CYP2C9*3* rs1057910 g.47639A>C	
Genotype	***CC***	110 (76.39)	***AA***	136 (91.89)
	CT	34 (23.61)	***AC***	12 (8.11)
	TT	0 (0.00)	***CC***	0 (0.00)
Allele	***C***	254 (88.19)	***A***	284 (95.95)
	T	34 (11.81)	***C***	12 (4.05)
HWE χ^2	**2.58**		**0.26**	

Tabl. 5. Allele and genotype frequencies of the studied polymorphic variants in the *VKORC1* gene in a group of population controls of Bulgarian origin

Total	**n=154**		**n=154**		**n=140**		**n=141**		**n=134**	
Variant	*VKORC1*2A* rs9934438 g.6399C>T		*VKORC1*2A* rs9923231 g.3588G>A		*VKORC1*2B* rs2884737 g.5723T>G		*VKORC1*3* rs7294 g.8956G>A		*VKORC1*4* rs17708472 g..5924C>T	
Genotype	***CC***	50 (32.47)	***GG***	50 (32.47)	***GG***	16 (11.43)	***GG***	59 (41.04)	***CC***	87 (64.93)
	CT	75 (48.70)	***GA***	75 (48.70)	***GT***	62 (44.29)	***GA***	68 (48.23)	***CT***	43 (32.09)
	TT	29 (18.83)	***AA***	29 (18.83)	***TT***	62 (44.29)	***AA***	14 (9.93)	***TT***	4 (2.99)
Allele	***C***	175 (56.82)	***G***	175 (56.82)	***G***	94 (33.57)	***G***	186 (65.96)	***C***	217 (80.97)
	T	133 (43.18)	***A***	133 (43.18)	***T***	186 (66.43)	***A***	96 (34.04)	***T***	51 (19.03)
HWE χ^2	**0.01**		**0.01**		**0.01**		**0.77**		**1.51**	

Table 6. Comparison of allele and genotype frequencies for polymorphic variant *VKORC1**2A (rs9934438) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	**n=154**	**n=246**	**n=181**	**n=286**	**n=85**	**n=97**	**n=379**	**n=93**	**n=89**	**n=14**	**n=89**	**n=55**	**n=98**
VKORC1*2A **rs9934438 g.6399C>T**	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
CC	50 (32.47)	217 (88.00)	56 (30.90)	0 (0.00)	31 (36.50)	0 (0.00)	138 (36.40)	43 (46.20)	32 (36.00)	4 (28.60)	0 (0.00)	16 (29.10)	28 (28.60)
CT	75 (48.70)	26 (11.00)	91 (50.30)	47 (16.00)	34 (40.00)	9 (9.30)	178 (47.00)	39 (41.90)	49 (55.10)	9 (64.30)	19 (21.30)	27 (49.10)	47 (48.00)
TT	29 (18.83)	3 (1.00)	34 (18.80)	239 (84.00)	20 (23.50)	88 (90.70)	63 (16.60)	11 (11.80)	8 (9.00)	1 (7.10)	70 (78.70)	12 (21.80)	23 (23.50)
C	175 (56.82)	460 (94.00)	203 (56.10)	47 (8.00)	96 (56.50)	9 (4.60)	454 (59.90)	125 (67.20)	113 (63.50)	17 (60.70)	19 (10.70)	59 (53.60)	103 (52.60)
T	133 (43.18)	32 (7.00)	159 (43.90)	525 (92.00)	74 (43.50)	185 (95.40)	304 (40.10)	61 (32.80)	65 (36.50)	11 (39.30)	159 (89.30)	51 (46.40)	93 (47.40)
P value	**-**	**<0,0001**	**0.88**	**<.0001**	**1**	**<.0001**	**0.39**	**0.02**	**0.15**	**0.84**	**<.0001**	**0.64**	**0.40**

Table 7. Comparison of allele and genotype frequencies for polymorphic variant *VKORC1**2B (rs2884737) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	**n=134**	**n=247**	**n=181**	**n=286**	**n=85**	**n=97**	**n=379**	**n=93**	**n=79**	**n=14**	**n=89**	**n=55**	**n=98**
VKORC1*2B **rs2884737 g.5723T>G**	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
GG	16 (11.43)	3 (1.20)	12 (6.60)	0 (0.00)	9 (10.60)	0 (0.00)	25 (6.60)	3 (3.20)	3 (3.40)	1 (7.10)	0 (0.00)	9 (16.40)	9 (9.20)
GT	62 (44.29)	4 (1.60)	54 (29.80)	2 (0.70)	29 (34.10)	2 (2.10)	141 (37.20)	29 (31.20)	34 (38.20)	6 (42.90)	0 (0.00)	21 (38.20)	43 (43.90)
TT	62 (44.29)	239 (97.20)	115 (63.50)	284 (99.30)	47 (55.30)	95 (97.90)	213 (56.20)	61 (65.60)	52 (58.40)	7 (50.00)	89 (100.00)	25 (45.50)	46 (46.90)
G	94 (33.57)	10 (2.00)	78 (21.50)	2 (0.30)	47 (27.60)	2 (1.00)	191 (25.20)	35 (18.80)	40 (22.50)	8 (28.60)	0 (0.00)	39 (35.50)	61 (31.10)
T	186 (66.43)	482 (98.00)	284 (78.50)	570 (99.70)	123 (72.40)	192 (99.00)	567 (74.80)	151 (81.20)	138 (77.50)	20 (71.40)	178 (100.00)	71 (64.50)	135 (68.90)
P value	**-**	**<0.0001**	**<0.001**	**<0.0001**	**0.21**	**<0.0001**	**0.007**	**<0.001**	**0.01**	**0.68**	**<0.0001**	**0.81**	**0.62**

Table 8. Comparison of allele and genotype frequencies for polymorphic variant *VKORC1**3 (rs7294) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	n=140	n=246	n=181	n=286	n=85	n=97	n=379	n=93	n=89	n=14	n=89	n=55	n=98
VKORC1*3 **rs7294 g.8956G>A**	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
GG	59 (41.04)	67 (27.20)	77 (42.50)	239 (83.60)	40 (47.10)	88 (90.70)	153 (40.40)	35 (37.60)	32 (36.00)	3 (21.40)	70 (78.70)	28 (50.90)	43 (43.90)
GA	68 (48.23)	122 (49.60)	81 (44.80)	47 (16.40)	38 (44.70)	9 (9.30)	183 (48.30)	40 (43.00)	48 (53.90)	10 (71.40)	19 (21.30)	19 (34.50)	47 (48.00)
AA	14 (9.93)	57 (23.20)	23 (12.70)	0 (0.00)	7 (8.20)	0 (0.00)	43 (11.30)	18 (19.40)	9 (10.10)	1 (7.10)	0 (0.00)	8 (14.50)	8 (8.20)
G	186 (65.96)	256 (52.00)	235 (64.90)	525 (91.80)	118 (69.40)	185 (95.40)	489 (64.50)	110 (59.10)	112 (62.90)	16 (57.10)	159 (89.30)	75 (68.20)	133 (67.90)
A	96 (34.04)	236 (48.00)	127 (35.10)	47 (8.20)	52 (30.60)	9 4.60()	269 (35.50)	76 (40.90)	66 (37.10)	12 (42.90)	19 (10.70)	35 (31.80)	63 (32.10)
P value	**-**	**<0.001**	**0.51**	**<0.0001**	**0.47**	**<0.0001**	**0.66**	**0.14**	**0.55**	**0.41**	**<0.0001**	**0.72**	**0.69**

Table 9. Comparison of allele and genotype frequencies for polymorphic variant *VKORC1**4 (rs17708472) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	n=141	n=246	n=181	n=286	n=85	n=97	n=379	n=93	n=89	n=14	n=89	n=55	n=98
VKORC1*4 **rs17708472 c.5924C>T**	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
CC	87 (64.93)	221 (89.80)	126 (69.60)	286 (100.00)	45 (52.90)	97 (100.00)	227 (59.90)	53 (57.00)	51 (57.30)	11 (78.60)	89 (100.00)	39 (70.90)	67 (68.40)
CT	43 (32.09)	25 (10.20	52 (28.70)	0 (0.00)	37 (43.50)	0 (0.00)	129 (34.00)	31 (33.30)	31 (34.80)	3 (21.40)	0 (0.00)	15 (27.30)	27 (27.60)
TT	4 (2.99)	0 (0.00)	3 (1.70)	0 (0.00)	3 (3.50)	0 (0.00)	23 (6.10)	9 (9.70)	7 (7.90)	0 (0.00)	0 (0.00)	1 (1.80)	4 (4.10)
C	217 (80.97)	467 (94.90)	304 (84.00)	0 (0.00)	127 (74.70)	0 (0.00)	583 (76.90)	137 (73.70)	133 (74.70)	25 (89.30)	0 (0.00)	93 (84.50)	161 (82.10)
T	51 (19.03)	25 (5.10)	58 (16.00)	572 (100.00)	43 (25.30)	194 (100.00)	175 (23.10)	49 (26.30)	45 (25.30)	3 (10.70)	178 (100.00)	17 (15.50)	35 (17.90)
P value	**-**	**<0.0001**	**0.34**	**<0.0001**	**0.12**	**<0.0001**	**0.20**	**0.07**	**0.13**	**0.32**	**<0.0001**	**0.46**	**0.81**

Table 10. Comparison of allele and genotype frequencies for polymorphic variant *CYP2C9**2 (rs1799853) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	n=144	n=246	n=281	n=286	n=85	n=97	n=379	n=93	n=89	n=14	n=89	n=55	n=98
CYP2C9*2 rs1799853 g.8633C>T	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
CC	110 (76.39)	237 (96.30)	137 (75.70)	284 (99.30)	65 (76.50)	97 (100.0)	294 (77.60)	76 (81.70)	74 (83.10)	10 (71.4)	89 (100.0)	37 (67.30)	69 (70.40)
CT	34 (23.61)	9 (3.70)	43 (23.80)	2 (0.60)	16 (18.18)	0 (0.00)	77 (20.30)	17 (18.30)	14 (15.70)	4 (28.60)	0 (0.00)	17 (30.90)	26 (26.50)
TT	0 (0.00)	0 (0.00)	1 (0.60)	0 (0.00)	4 (4.70)	0 (0.00)	8 (2.10)	0 (0.00)	1 (1.10)	0 (0.00)	0 (0.00)	1 (1.80)	3 (3.10)
C	254 (88.19)	483 (98.20)	317 (87.60)	570 (99.70)	146 (85.90)	194 (100.0)	665 (87.70)	169 (90.90)	162 (91.00)	24 (85.70)	178 (100.0)	91 (82.70)	164 (83.70)
T	34 (11.81)	9 (1.80)	45 (12.40)	2 (0.30)	24 (14.10)	0 (0.00)	93 (12.30)	17 (9.10)	16 (9.00)	4 (14.30)	0 (0.00)	19 (17.30)	32 (16.30)
P value	**-**	**<.0001**	**0.81**	**<.0001**	**0.56**	**<.001**	**0.92**	**0.45**	**0.36**	**0.76**	**<.0001**	**0.19**	**0.18**

Table 11. Comparison of allele and genotype frequencies for polymorphic variant *CYP2C9**3 (rs1057910) in the group of population controls of Bulgarian origin with results obtained for other ethnic groups

Total	**n=148**	**n=246**	**n=181**	**n=286**	**n=85**	**n=97**	**n=379**	**n=93**	**n=89**	**n=14**	**n=89**	**n=55**	**n=98**
CYP2C9*3 **rs1057910 g.47639A>C**	**BG**	**AFR**	**AMR**	**ASN**	**CEU**	**CHB**	**EUR**	**FIN**	**GBR**	**IBS**	**JPT**	**PUR**	**TSI**
AA	136 (91.89)	243 (98.80)	160 (88.40)	264 (92.30)	76 (89.40)	89 (91.80)	333 (87.90)	82 (88.20)	79 (88.80)	11 (78.60)	85 (95.50)	51 (92.70)	85 (86.70)
AC	12 (8.11)	3 (1.20)	21 (11.60)	21 (7.30)	9 (10.60)	8 (8.20)	46 (12.10)	11 (11.80)	10 (11.20)	3 (21.40)	4 (4.50)	4 (7.30)	13 (13.30)
CC	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.30)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
A	284 (95.95)	489 (99.40)	341 (94.20)	549 (96.00)	161 (94.70)	186 (95.90)	712 (93.90)	175 (94.10)	168 (94.40)	25 (89.30)	174 (97.80)	106 (96.40)	183 (93.40)
C	12 (4.05)	3 (0.60)	21 (5.80)	23 (4.00)	9 (5.30)	8 (4.10)	46 (6.10)	11 (5.90)	10 (5.60)	3 (10.70)	4 (2.20)	4 (3.60)	13 (6.60)
P value	**-**	**0.002**	**0.37**	**1**	**0.64**	**1**	**0.25**	**0.38**	**0.50**	**0.13**	**0.31**	**1**	**0.21**

Legend: **AFR** – African, **AMR** – Ad Mixed American, **ASN** – Asian, **EUR** – European, **BG** – Bulgarian, **CHB** – Han Chinese in Bejing, China, **JPT** – Japanese in Tokyo, Japan, **CEU** – Utah Residents (CEPH) with Northern and Western European ancestry, **TSI** – Toscani in Italia, **FIN** – Finnish in Finland, **GBR** – British in England and Scotland, **IBS** – Iberian population in Spain, **PUR** – Puerto Ricans from Puerto Rico

No statistically significant differences were observed between the distributions of the studied polymorphic variants in the Bulgarian population and those in Europeans.

DISCUSSION

In the current study, we evaluated the prevalence of selected polymorphic variants in the *VKORC1* and *CYP2C9* genes in individuals of Bulgarian origin. The reported polymorphisms are located within genes whose protein products are involved in the metabolism of the indirect anticoagulant acenocoumarol.

Acenocoumarol (AC) is a 4-hydroxycoumarin derivate that is widely used for both primary and secondary prevention of various thromboembolic conditions, such as arterial and venous thromboembolism, atrial fibrillation, ischemic stroke, aortic or mitral valve replacement and cardiac arrhythmia in many European countries, such as in Spain, the Netherlands, Italy and France [7], also in Bulgaria.

The disadvantages of therapy with this medication are its narrow therapeutic range and the large inter-individual variability in the treatment response. Many efforts have been made to improve treatment with coumarin anticoagulants, but the risk of complications remains significant. Currently, INR measurement is the only laboratory test used for monitoring the anticoagulation effect of acenocoumarol.

There is wide variability in the response to therapy with this medication because of the influence of environmental factors, age, gender, race, other medication intake, liver and thyroid disease, CAD, fever, malignancy, and finally genetic factors, making it difficult to determine the optimal dose of the anticoagulant [8].

It is well established that polymorphic variants in the *CYP2C9* (cytochrome P450 2C9) and *VKORC1* (vitamin K epoxide reductase complex subunit 1) genes have a significant impact on the maintenance dose of coumarin anticoagulants. The *VKORC1* gene encodes subunit 1 of the vitamin K epoxide reductase complex (*VKORC1*), an enzyme that is involved in the vitamin K cycle and metabolism. The *CYP2C9* gene encodes an enzyme of the cytochrome P450 group (cytochrome P450 2C9). Polymorphic variants in both these genes are responsible for over 30% of individual variation in acenocoumarol dosage. Other important contributing factors are patient age, diet, height and weight, potential drug interactions such as concomitant administration of amiodarone and other drugs that may increase or decrease the active effects of acenocoumarol. Co-existence of *CYP2C9**2/*3, *VKORC1* 1173TT and *VKORC1-1639AA* genotypes are associated with an increased risk of thrombosis. These patients require extremely low doses of anticoagulant, and they also have a significantly increased risk of bleeding [9].

The co-existence of *CYP2C9**2/*3, *VKORC1 1173TT*, and *VKORC1-1639AA* genotypes is associated with

an increased risk of thrombosis due to their impact on warfarin metabolism and anticoagulation efficacy. These genotypes influence the levels of vitamin K-dependent clotting factors, which are crucial for maintaining hemostasis.

The *CYP2C9**2 and *3 alleles are linked to reduced enzyme activity, leading to higher acenocoumarol sensitivity and increased risk of bleeding or thrombosis if not properly dosed. The *VKORC1-1639AA* genotype results in lower VKOR enzyme levels, which can lead to inadequate anticoagulation and a higher risk of thrombotic events. Studies show that patients with *VKORC1-1639AA* and *CYP2C9**3 genotypes have poorer anticoagulation control, increasing the likelihood of thromboembolic complications [10].

Conversely, while these genotypes are associated with increased thrombosis risk, some studies suggest that careful monitoring and individualized dosing can mitigate these risks, emphasizing the need for personalized medicine in anticoagulation therapy.

The *CYP2C9* gene is localized on chromosome 10 (10q24.2). It covers a region of 55 kb and contains 9 exons translated into a protein of 490 amino acids (AA). The gene is highly polymorphic. To date, more than 30 non-synonymous polymorphic variants have been described, showing high inter-ethnic variability in frequency. The most frequent allele is *CYP2C9**1 (wild type) and dominates in all ethnic groups. In Caucasian populations, two other common alleles are *CYP2C9**2 (rs1799853, 430C>T, 8-19%) and *CYP2C9**3 (rs1057910, 1075A>C, 4-16%). Both variants are localized in the coding region of the gene and result in an amino acid substitution in the isoenzyme, which decreases its metabolic capacity towards its substrates. Some more recently identified polymorphisms in this gene resulting in decreased (*CYP2C9**5, *CYP2C9**11) or absence (*CYP2C9**6) of enzyme activity have been identified in African Americans with low allele frequencies [8].

Vitamin K epoxide reductase (VKOR) represents a molecular target for the actions of indirect anticoagulants; therefore, the identification and molecular characterization of the gene encoding VKOR (*VKORC1*) is a milestone in the elucidation of the pharmacogenetic basis of oral anticoagulant therapy [11]. The enzyme is implicated in the vitamin K cycle, and genetic variants of *VKORC1* gene are associated with deficiency of the vitamin K-dependent clotting factors (II, IIV, IX, and X) of the coagulation cascade and increased sensitivity to coumarin anticoagulants. Several studies have elucidated the impact of these variants and the response to acenocoumarol drug therapy. The polymorphic variant -1639 G>A in the gene promoter is associated with a low dose requirement of acenocoumarol [12-16].

Carriers of the polymorphic allele for this marker require a significantly higher optimal maintenance anticoagulant dose needed to achieve target INR in the range of 2.0 to 4.5. Over the last few years, this association has been identified and subsequently confirmed in different populations, and the implications of these polymorphisms with respect to optimal anticoagulant dose have varied across studies [12-16]. The polymorphic variants 1173C>T in intron 1 and 3730 G>A in the 3' UTR of *VKORC1* were detected in linkage disequilibrium with -1639 G>A in the gene promoter, accounting for the requirement of a low anticoagulant dose [134]. Therefore, *VKORC1* -1639 G>A has become the polymorphic variant with the most importance in terms of pharmacogenetic response to coumarin anticoagulant therapy. Reduced VKOR activity was found to be associated with the carrying of this particular polymorphic variant [11].

In addition to the polymorphic variant in the promoter region of the *VKORC1* gene (-1639 G>A), those in the non-coding regions also contribute significantly to determining the maintenance dose of warfarin and acenocoumarol. It was also found that haplotypes of the *VKORC1* gene were associated with high variation in the required dose of anticoagulants. In previous studies, haplotypes 2B, 3 and 4 have been considered, with the carriers of these variants required higher doses of medication compared to wild-type carriers [17-19].

In the study of Velizarova el al. the calculated frequency of *CYP2C9**1 allele was 74.25%, *CYP2C9**2 allele was 13%, and *CYP2C9**3 allele was 12.75%, and all allelic frequencies were in Hardy-Weinberg equilibrium (p-value = 0.358). The major *VKORC1* genotype was G/A – 47%, followed by G/G – 35.5% and A/A – 17.5%). Based on Hardy–Weinberg Equilibrium, there was no significant difference between observed and expected frequencies (X = -3.779), presumably because of the homogeneity in the population. Their results demonstrated good agreement with the results obtained in other studies conducted in the Caucasian population, also with this in our study [20].

Also, the results for *CYP2C9**2 and *CYP2C9**3 variants in our study were similar to those reported by Saraeva et al. in 2007 [*CYP2C9**2 (0.12 vs 0.16) and *CYP2C9**3 (0.8 vs 0.6) [8].

In a study by Jakjovski et al., frequency of alleles of studied polymorphic variants in *CYP2C9* and *VKORC1* genes varied 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. The test of neutrality showed significant negative value for *VKORC1* polymorphism that indicates balancing selection operating on the alleles at that locus [21].

The investigation of Giraldo-Ocampo et al. aimed to determine the frequency of the polymorphisms in the *VKORC1*, *CYP2C9*, and *CYP4F2* genes in healthy individuals from Cali, Colombia. *CYP2C9**2, *CYP2C9**3, *CYP2C9**8, *CYP2C9**9, *CYP2C9**11, *CYP4F2**3, rs12777823, and *VKORC1**2 were the alleles found by Sanger sequencing in the 107 subjects included in this study. The 2 most common polymorphisms found were c.-1639G > A (*VKORC1**2) in 80 (74.8%) participants (n = 53 [49.5%] and n = 27 [25.2%] in heterozygosis and homozygosis, respectively) and the variant c.1297G > A (*CYP4F2**3) in 49 (45.8%) subjects (n = 41 [38.3%] heterozygous and n = 8 [7.8%] homozygous). Among the *CYP2C9* alleles, c.430C > T (*CYP2C9**2) was the most frequent variant found in 16 individuals (14%) of whom 15 were carriers and only one in a homozygotic state [22].

In the study of Zhou et al., the authors observed heterogeneity between allele frequencies of countries within the same macrogeographical region. The frequency of *CYP2C9**2 in Turkey (10.5%) were considerably lower than in its neighboring countries Bulgaria (12.5%), Greece (12.9%), Lebanon (15.4%) and Iran (18.1%). The frequency of *CYP2C9**3 allele in Bulgarian was reported as 7.5%. Reduced *CYP2C9* activity is most prevalent in South Europe and the Middle East, as well as in specific founder populations in Southeast Asia [23].

CONCLUSION

This study established the population frequencies of polymorphic variants in *CYP2C9* gene (*CYP2C9**2, 430C>T, rs1799853; and *CYP2C9**3, 1075A>C, rs1057910) and *VKORC1* gene (*VKORC1**2A, rs9923231, g. 3588 G>A; rs9934438, g.6399 C>T; *VKORC1**2B, rs2884737, g.5723T>G; *VKORC1**3, rs7294, g.8956 G>A; *VKORC1**4, rs17708472, g.5924C>T). There were no disagreements between the allele and genotype frequencies of the polymorphisms that were reported in this study and those published for other European populations.

Furthermore, the methods introduced in the current study for the genetic analysis of allelic variants in the *CYP2C9* and *VKORC1* genes, which are important for the response to acenocoumarol, are highly specific and generate reproducible results. High-resolution DNA melting is a methodology that has been applied in laboratory and clinical practice over the last few years.

Conflict of Interest Statement: *The authors declare no conflicts of interest related to this work.*

Funding: *This work was supported through grants funded by the Ministry of Education and Science (MES) of Republic of Bulgaria/Contracts D01-165/2022 (D01-138/2024) and D01-371/2023/, within National Roadmap for Research Infrastructure 2020-2027 and by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project No. BG-RRP-2.004-0003.*

Ethical statement: *This study has been performed in accordance with the ethical standards as laid down in the Declaration of Helsinki. The observational study was approved by the Ethics Committee of the Medical University – Sofia.*

Informed Consent from Participants*: Informed consent was obtained from all participants included in the study.*

REFERENCES

1. Rakićević L, Kovač M, Radojković D, Radojković M. The ‚*VKORC1*‘ and ‚*CYP2C9*‘ gene variants as pharmacogenetic factors in acenocoumarol therapy in Serbian patients: Consideration of hypersensitivity and resistance. Srpski arhiv za celokupno lekarstvo. 2022;150(3-4):156-62. doi: 10.2298/SARH211118013R
2. Roco A, Nieto E, Suarez M, et al. A Pharmacogenetically Guided Acenocoumarol Dosing Algorithm for Chilean Patients: A Discovery Cohort Study. Front Pharmacol. 2020;11:325.
3. Tong HY, Borobia AM, Quintana-Diaz M, et al. Acenocoumarol Pharmacogenetic Dosing Algorithm versus Usual Care in Patients with Venous Thromboembolism: A Randomised Clinical Trial. J Clin Med. 2021;10(13).
4. Cullell N, Carrera C, Muino E, et al. Genome-Wide Association Study of *VKORC1* and *CYP2C9* on acenocoumarol dose, stroke recurrence and intracranial haemorrhage in Spain. Sci Rep. 2020;10(1):2806.
5. Ragia G, Kolovou V, Kolovou G, et al. A novel acenocoumarol pharmacogenomic dosing algorithm for the Greek population of EU-PACT trial. Pharmacogenomics. 2017;18(1):23-34.
6. Gonzalez-Covarrubias V, Urena-Carrion J, Villegas-Torres B, et al. Pharmacogenetic Variation in Over 100 Genes in Patients Receiving Acenocumarol. Front Pharmacol. 2017;8:863.
7. Pengo V, Pegoraro C, Cucchini U, Iliceto S. Worldwide management of oral anticoagulant therapy: the ISAM study. J Thromb Thrombolysis. 2006;21(1):73-7.
8. Schalekamp T, de Boer A. Pharmacogenetics of oral anticoagulant therapy. Curr Pharm Des. 2010;16(2):187-203.
9. Kovac MK, Rakicevic LB, Radojkovic DP. Extreme sensitivity to acenocoumarol therapy in patient with both VKORC.-1639 A/A and *CYP2C9**1/*3 genotypes. J Thromb Thrombolysis. 2011;32(3):368-71.
10. Sun B, Ma S, Xiao F, et al. Integrated analysis of clinical and genetic factors on the interindividual variation of warfarin anticoagulation efficacy in clinical practice. BMC Cardiovasc Disord. 2023;23(1):279.
11. Rathore SS, Agarwal SK, Pande S, et al. Pharmacogenetic aspects of coumarinic oral anticoagulant therapies. Indian J Clin Biochem. 2011;26(3):222-9.

12. Krishna Kumar D, Madhan S, Balachander J, et al. Effect of *CYP2C9* and *VKORC1* genetic polymorphisms on mean daily maintenance dose of acenocoumarol in South Indian patients. Thromb Res. 2013;131(4):363-7.
13. Cerezo-Manchado JJ, Rosafalco M, Anton AI, et al. Creating a genotype-based dosing algorithm for acenocoumarol steady dose. Thromb Haemost. 2013;109(1):146-53.
14. Borobia AM, Lubomirov R, Ramirez E, et al. An acenocoumarol dosing algorithm using clinical and pharmacogenetic data in Spanish patients with thromboembolic disease. PLoS One. 2012;7(7):e41360.
15. Bazan NS, Sabry NA, Rizk A, et al. Validation of pharmacogenetic algorithms and warfarin dosing table in Egyptian patients. Int J Clin Pharm. 2012;34(6):837-44.
16. Buzoianu AD, Militaru FC, Vesa SC, et al. The impact of the *CYP2C9* and *VKORC1* polymorphisms on acenocoumarol dose requirements in a Romanian population. Blood Cells Mol Dis. 2013;50(3):166-70.
17. Rieder MJ, Reiner AP, Gage BF, et al. Effect of *VKORC1* haplotypes on transcriptional regulation and warfarin dose. N Engl J Med. 2005;352(22):2285-93.
18. Rieder MJ, Reiner AP, Rettie AE. Gamma-glutamyl carboxylase (GGCX) tagSNPs have limited utility for predicting warfarin maintenance dose. J Thromb Haemost. 2007;5(11):2227-34.
19. Verde Z, Ruiz JR, Santiago C, et al. A novel, single algorithm approach to predict acenocoumarol dose based on *CYP2C9* and *VKORC1* allele variants. PLoS One. 2010;5(6):e11210.
20. Velizarova M, Abedinov P, Svinarov D, et al. Frequency Distribution of *CYP2C9* and *VKORC1* Mutations among Bulgarian Patients and their Importance for Anticoagulant Therapy. Clin Lab. 2022;68(12).
21. Jakjovski K, Labachevski N, Petlichkovski A, et al. Distribution of *CYP2C9* and *VKORC1* Gene Polymorphisms in Healthy Macedonian Male Population. Macedonian J Med Sciences. 2014;6(4):5.
22. Giraldo-Ocampo S, Diaz-Ordonez L, Silva-Cuero YK, et al. Frequency of polymorphisms in the *CYP2C9*, *VKORC1*, and *CYP4F2* genes related to the metabolism of Warfarin in healthy donors from Cali, Colombia. Medicine (Baltimore). 2023;102(30):e34204.
23. Zhou Y, Nevosadova L, Eliasson E, Lauschke VM. Global distribution of functionally important *CYP2C9* alleles and their inferred metabolic consequences. Hum Genomics. 2023;17(1):15.