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ORIGINAL ARTICLE

ESTABLISHMENT OF ACENOCOUMAROL PHARMACOGENETIC ALGORITHM INCLUDING CYP2C9 AND VKORC1 GENOTYPES IN BULGARIAN PATIENTS TREATED WITH COUMARIN ANTICOAGULANTS

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Abstract. Introduction: *Acenocoumarol, a 4-hydroxycoumarin derivative, is widely prescribed for the primary and secondary prevention of thromboembolic disorders. Maintenance dosing of acenocoumarol is significantly influenced by polymorphic variants in the CYP2C9 and VKORC1 genes. Other critical factors affecting dosing include patient age, diet, body height and weight, and potential drug interactions, particularly with concurrent use of medications such as amiodarone and statins.* ***Objectives:*** *The primary goal of this investigation is to develop a pharmacogenetic dosing algorithm for acenocoumarol based on CYP2C9 and VKORC1 genotypes in Bulgarian patients.* ***Methods:*** *A total of 120 patients, aged 18 to 70 years, undergoing stable acenocoumarol therapy were enrolled in this study. DNA was extracted using the Chemagic Magnetic Separation Module I (Chemagen AG) following the manufacturer's protocol, at the Molecular Medicine Center, Medical University – Sofia, Bulgaria. To develop the final clinical and pharmacogenetic dosing algorithms, variables such as age, gender, diagnosis, weight, amiodarone use, and genotypes (CYP2C9*2, CYP2C9*3, and VKORC1-1639G>A) were incorporated into multiple linear regression (MLR) model.* ***Results:*** *For the analysis, we conducted genotyping of ten polymorphic variants across four genes relevant to acenocoumarol response: CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057310), VKORC1*2A (rs9923231 and rs9934438), VKORC1*2B (rs2884737), VKORC1*3 (rs7294), VKORC1*4 (rs17708472), and APOE (rs7412 and rs429358). Single-component and multiple linear regression analyses were applied to evaluate both genetic and non-genetic factors and their effects on the daily acenocoumarol dose in the patient cohort. The resulting mathematical dosing algorithm is provided below:* ***Optimal daily maintenance dose of acenocoumarol*** *= 5.939 – 0.033*(age in years) – 1.149* (number of VKORC1*2A alleles) + 0.433*(number of VKORC1*3 alleles) – 1.425*(number of CYP2C9*2 alleles) – 0.486*(number of CYP2C9*3 alleles).* ***Conclusion:*** *The multivariate analysis revealed that age and the presence of CYP2C9*2, CYP2C9*3, VKORC1*2A, and VKORC1*3 alleles accounted for 43.8% of the variation in the average daily maintenance dose of acenocoumarol.*

Key words: *acenocoumarol, CYP2C9, VKORC1, algorithm*

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INTRODUCTION

Acenocoumarol, a 4-hydroxycoumarin derivative, is widely used for primary and secondary prevention of various thromboembolic disorders, such as arterial and venous thromboembolism, ischemic stroke, aortic or mitral heart valve replacement and cardiac arrhythmia in many European countries [1], as well as in Bulgaria. The disadvantage of therapy with this medication is its narrow therapeutic range and the large inter-individual variability in treatment response. There are many efforts to improve the treatment with coumarin anticoagulants, but the risk of complications remains significant. At present, anticoagulant monitoring is accomplished by INR measurements.

Acenocoumarol is a vitamin K antagonist, a fat-soluble vitamin required for the activation of the main blood clotting factors in the coagulation cascade (factors II, VII, IX, and X). These factors, glycoproteins containing glutamic acid (Glu) residues, undergo γ-carboxylation, which converts Glu residues to γ-carboxyglutamic (γ-carboxy-Glu) residues. Calcium binding to these residues induces conformational changes necessary for their function within the coagulation cascade. This γ-carboxylation step is catalyzed by the vitamin K-dependent enzyme γ-glutamylcarboxylase (GGCX), with the reduced form of vitamin K (vitamin K hydroquinone) acting as a cofactor. During carboxylation, vitamin K hydroquinone is oxidized to vitamin K-2,3-epoxide, which is then rapidly reduced to prevent excessive tissue accumulation of vitamin K. The initial reduction step is catalyzed by the vitamin K epoxide reductase complex (VKOR) [1].

All coumarin derivatives inhibit VKOR, forming the basis of their anticoagulant effects. This inhibition depletes the reduced form of vitamin K (vitamin KH2), essential for carboxylating the N-terminal glutamate residues of vitamin K-dependent coagulation factors. Consequently, the carboxylation and activation of these factors are limited. This process inhibits the synthesis of coagulation factors II, VII, IX, and X, along with anticoagulant proteins C and S, leading to prolonged prothrombin time and reduced thrombogenicity of blood clots. The full anticoagulant effect of coumarins emerges over several days, as the reduction of clotting factors to target levels depends on their half-lives, which range from approximately 6 hours for factor VII to 60 hours for prothrombin [1].

The optimal maintenance dose of acenocoumarol is determined by both genetic and non-genetic factors. It is well known that polymorphic variants in CYP2C9 and VKORC1 genes significantly affect the maintenance acenocoumarol dose. VKORC1 encoding subunit 1 of vitamin K epoxide reductase complex (VKORC1) – an enzyme involved in vitamin K metabolism. CYP2C9 is an enzyme of cytochrome P450 superfamily (2C9 cytochrome P450). Polymorphic variants in these genes are responsible for over 30% of individual variations in acenocoumarol dosage. Other important factors are age, diet, height and weight, potential drug interactions, such as concomitant administration of amiodarone and statins [2].

In recent years, several pharmacogenetic algorithms for determining the optimal acenocoumarol dosage have been established for different population groups. All models incorporate both clinical variables – such as age, sex, body mass index (BMI), concomitant medications, and vitamin K intake – and genetic factors, including polymorphic variants in the CYP2C9, VKORC1, APOE, and CYP4F2 genes. Eight distinct mathematical algorithms, tailored to specific population groups and reported in the medical literature, were developed by the following research groups: Markatos et al., Van Schie et al., Borobia et al., Rathore et al., Serezo-Manchado et al., Kumar et al., Pop et al., and Wolkanin-Bartnik et al. [3-10].

In our previous study we also published a mathematical model based on clinical factors and CYP2C9*2,*3, VKORC1-1639G>A polymorphisms for Bulgarian patients with increase thrombosis risk. It was found that VKORC-1639G>A (25.5%), CYP2C9*2 (7.8%), CYP2C9*3 (6.1%), age (13.6%), and diagnosis (6.0%) significantly affected acenocoumarol dose variability. These factors with additional factors, such as sex (0.1%, p = 0.76), weight (2.6%, p = 0.14) and amio-

darone use (3.0%, p = 0.059) accounted for 46.5% and 23.0% of the dose variability for genetic and clinical algorithm, respectively [11].

MATERIALS AND METHODS

Characteristics of the studied patients

A total of 120 patients, aged 18 to 70 years, undergoing stable acenocoumarol therapy from various regions of Bulgaria were enrolled in this study. This cohort was utilized to develop dose prediction models for determining the initial anticoagulant dose at the start of therapy. All participants were referred to the outpatient clinics at St. Ekaterina University Hospital, Sofia, for acenocoumarol therapy monitoring.

Inclusion criteria required participants to have a diagnosis of deep venous thrombosis (DVT), pulmonary embolism (PE), atrial fibrillation (AF), heart valve replacement (HVR), or other cardiovascular conditions and to be on a stable acenocoumarol dose. Each patient had been receiving an indirect anticoagulant for a minimum of 3 months and had reached a stable, optimal dose of acenocoumarol, with a target international normalized ratio (INR) between 2 and 4, adjusted based on the clinical indication for anticoagulation.

The INR target range of 2 to 4 was established according to clinical practice and in consultation with leading clinical laboratory specialists at the University Multiprofile Hospital for Active Treatment “St. Ekaterina" in Sofia, Bulgaria. Exclusion criteria included liver disease, thyroid dysfunction, active cancer diagnosis, alcohol or drug abuse, suspected pregnancy, and lack of informed consent. Clinical data for each participant – including diagnosis, ethnicity, age, height, weight, mean acenocoumarol dose, INR value, co-morbidities, and co-medications – were collected from patient case records.

All subjects were ethnic Bulgarians (Caucasians). This investigation received approval from the Ethics Committees of the Medical University of Sofia and the Medical University of Pleven, Bulgaria, and was conducted by the ethical standards outlined in the Declaration of Helsinki.

DNA isolation and SNP genotyping

Peripheral blood samples from each participant were collected in Vacutainers containing EDTA (ethylenediaminetetraacetic acid) and stored at 4°C prior to DNA isolation. The INR value was measured using the Normotest® (Technoclone GmbH) from capillary blood samples. DNA extraction was performed using the Chemagic Magnetic Separation Module I (Chemagen AG) following the manufacturer’s protocol at the Molecular Medicine Center, Medical University of Sofia, Bulgaria.

The polymorphisms analyzed included CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057310), VKORC1*2A (rs9923231 and rs9934438), VKORC1*2B (rs2884737), VKORC1*3 (rs7294), VKORC1*4 (rs17708472), and APOE (rs7412 and rs429358). Genotyping was conducted using High-Resolution Melting Analysis (HRMA) on a RotorGene 6000 (Qiagen). The primer sequences and cycling conditions are available upon request. All genotypes were verified by direct sequencing of randomly selected samples.

Statistical analysis

The Hardy-Weinberg equilibrium was verified for all investigated polymorphic variants by the χ^2 test. A $p < 0.05$ was considered statistically significant. Linkage disequilibrium (LD) pattern and haplotype frequencies were estimated for – 1639G>A and 1173C>T polymorphisms in VKORC1 gene.

The mean daily maintenance doses of acenocoumarol were compared between the different genotype groups in the derivation cohort (DC) for all tested polymorphic variants using the t-test. Numerical data were presented as mean ± standard deviation when parametric and as median (25th-75th percentiles) when non-parametric.

We selected univariate and multivariate linear regression (ULR, MLR) analyses to evaluate major genetic and non-genetic factors and their influence on the maintenance daily dose of acenocoumarol in DC. For generation of the final best clinical and pharmacogenetic algorithms, variables such as age, gender, diagnosis, weight, amiodarone use, CYP2C9*2, CYP2C9*3 and VKORC1 -1639G>A genotypes were included in MLR. The clinical factors were similar in the development of both models. The genetic factors were added to clinical models to develop a pharmacogenetic algorithm. B-coefficients provided the relative power of each variable included in the models. R2 is the percentage of the dose variability explained by the relevant model. The robustness of the determined MLR parameters was evaluated by ten-fold repetition of the process with randomization of the derivation and testing cohorts used.

All statistical analysis was performed using the Statistical Package for Social Sciences for Windows (SPSS ver. 19; SPSS, Chicago, IL) and Excel 2010 (Windows 2010).

RESULTS

Clinical and demographic characteristics of the total sample of Bulgarian patients undergoing aceno-

coumarol anticoagulant therapy (N=120) are presented in Table 1.

Table 1. Clinical and demographic characteristics of patients included in the studied group

Characteristics	Derivation cohort (n = 120) (mean±SD)
Age (years) (mean±SD)	58.04 ± 14.13
Male [n (%)]	66 (55.00%)
Female [n (%)]	54 (45.00%)
Ethnicity	Caucasian
Heigh (sm) (mean±SD)	172.63 ± 9.74
Weight (kg) (mean±SD)	80.90 ± 17.08
BMI (kg/m^2) (mean±SD)	27.96 ± 5.35
Average acenocoumarol dose	3.02 ± 1.96
Amiodarone administration	12 (10.17%)
Atrial fibrillation	27 (22.50)
Pulmonary thromboembolism (PT)	9 (7.50)
Deep venous thrombosis (DVT)	11 (9.17)
Heart valve replacement (HVR)	54 (45.00)
Others	13 (15.83)

* Mann-Whitney test; ** Chi-square test

For the purpose of the intended analysis, genotyping was conducted for ten polymorphic variants across four genes: CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057310), VKORC1*2A (rs9923231 and rs9934438), VKORC1*2B (rs2884737), VKORC1*3 (rs7294), VKORC1*4 (rs17708472), and APOE (rs7412 and rs429358). These variants are considered relevant to the response to acenocoumarol therapy.

The allelic frequencies of CYP2C9*1 (76.47%), CYP2C9*2 (15.29%), and CYP2C9*3 (8.24%) are consistent with those previously reported for the Bulgarian population in a study by Saraeva et al. [21]. The frequency of the A allele for the polymorphic variant VKORC1 -1639 G>A was found to be 45.59%.

For the polymorphic variant CYP2C9*3, there was no deviation from Hardy-Weinberg equilibrium (χ^2 = 0.06, p = 0.80). Similar results were observed for the following variants: VKORC1*3 (p = 1.0, χ^2 = 2.60), VKORC1*4 (p = 1.0, χ^2 = 0.0), APOE*2 (χ^2 = 0.45, p = 0.50), and APOE*4 (χ^2 = 0.21, p = 0.65).

Differences in Hardy-Weinberg equilibrium were observed for other polymorphic variants: VKORC1*2A (χ^2 = 7.18, p = 0.007), VKORC1*2B (χ^2 = 12.8, p = 0.0003), and CYP2C9*2 (χ^2 = 4.59, p = 0.03). These deviations could be due to the higher proportion of patients in the sample requiring low acenocoumarol doses. This imbalance may result in a higher frequency of polymorphic alleles associated with a significant reduction in optimal maintenance doses of anticoagulants, which is an acceptable deviation from Hardy-Weinberg equilibrium in this context.

Genotyping was also performed for 140 healthy control subjects to establish the population frequency of the studied variants in the CYP2C9 and VKORC1 genes for the Bulgarian population. No deviations from Hardy-Weinberg equilibrium were observed for any of the genetic markers in this control group.

The purpose of this study was to assess the impact of polymorphic variants in the CYP2C9, VKORC1, and APOE genes on the response to acenocoumarol therapy and to develop a mathematical model for determining the optimal anticoagulant dose. The algorithm was constructed based on the clinical and genetic data of patients in the main test group (N = 120).

It is important to note that a small number of patients in the test group had missing genotypes due to unsuccessful genotyping. To avoid any bias in the statistical analysis, patients with missing data for a specific factor were automatically excluded from analyses involving that factor. The results of the nonparametric correlation analysis, which examined associations between clinical, demographic, and genetic characteristics and the daily dose of acenocoumarol, are summarized in Table 2.

Spearman's rho correlation analysis show that age (r^2 = -0.273, p = 0.003 and genetic variants in CYP2C9 and VKORC1 genes [CYP2C9*2 (r^2 = -0.280, p = 0.002), CYP2C9*3 (r^2 = -0.193, p = 0.036), VKORC1*2A (r^2 = -0.545, p < 0.001), VKORC1*2B (r^2 = 0.398, p < 0.001), VKORC1*3 (r^2 = 0.416, p < 0.001) and VKORC1*4 (r^2 = 0.208, p = 0.026)] significantly affect the average daily acenocoumarol maintenance dose in studied group of Bulgarian patients with increased thrombosis risk (Table 2).

Other factors, such as gender (r^2 = -0.052, p = 0.53), diagnosis (r^2 = 0.063, p = 0.50), height (r^2 = 0.150, p = 0.25), weight (r^2 = 0.170, p = 0.10), BMI (r^2 = 0.259, p = 0.46), amiodarone administration (r^2 = 0.125, p = 0.21), smoking status (r^2 = 0.041, p = 0.69), statin administration (r^2 = 0.139, p = 0.21), and the APOE variants rs7412 (r^2 = 0.076, p = 0.43) and rs429358 (r^2 = -0.069, p = 0.48) showed no association with the required anticoagulant dose.

Our study of the VKORC1-1639G>A (rs9934438) and VKORC1 1173C>T (rs9923231) polymorphic variants, which together define the VKORC1*2A haplotype, confirmed their association with a need for lower doses of indirect anticoagulants in carriers of a polymorphic allele – especially in those homozygous for the allele. Additionally, we found that these two

Table 2. Correlation analysis of the relationship of studied polymorphic variants in CYP2C9 and VKORC1 genes to the average acenocoumarol maintenance dose in patient's groups with an increased thrombosis risk.

Characteristics Correlation coefficient		Kendall's tau_b		Spearman's rho	
		P value	Correlation coefficient	P value	
Clinical	Diagnosis	0.055	0.45	0.063	0.50
	Gender	-0.043	0.58	-0.052	0.58
	Age	-0.190	0.003	-0.273	0.003
	Weight	0.114	0.11	0.170	0.102
	Heigh	0.105	0.26	0.150	0.25
	BMI	0.192	0.035	0.259	0.46
	Cigarette smoking	0.034	0.69	0.041	0.69
	Amiodarone	0.105	0.20	0.125	0.21
	Statins	0.118	0.20	0.139	0.21
Pharmacogenetic	*VKORC1*2A* rs9923231 (-1639G>A)	-0.446	< 0.001	-0.545	< 0.001
	*VKORC1*2B* rs2884737	0.321	< 0.001	0.398	< 0.001
	*VKORC1*3* rs7294	0.343	< 0.001	0.416	< 0.001
	*VKORC1*4* rs17708472	0.174	0.023	0.208	0.026
	*CYP2C9*2*	-0.235	0.002	-0.280	0.002
	*CYP2C9*3*	-0.162	0.037	-0.193	0.036
	APOE rs7412	0.064	0.43	0.076	0.43
	APOE rs429358	-0.057	0.48	-0.068	0.48

VKORC1 variants are in complete linkage disequilibrium, consistent with findings from other studies [4, 5]. Consequently, only one of these polymorphisms, VKORC1-1639G>A, was included in subsequent analyses. Using the t-test, we compared mean daily maintenance doses of acenocoumarol across patient groups with different genotypes in the test group for all studied polymorphic variants. Numerical data are reported as mean ± standard deviation.

Genotypic and allelic frequencies of the studied polymorphisms are given in Table 3.

The mean daily maintenance dose of acenocoumarol in patients carrying any polymorphic variant was significantly lower than the mean dose required for patients with the wild-type genotype. Patients with wild-type genotypes for CYP2C9 and VKORC1 required similar daily maintenance doses of acenocoumarol (3.51 ± 2.00 mg for CYP2C9*1/*1 and 4.01 ± 1.89 mg for VKORC1*1/*1). Carriers of the CYP2C9*1/*2 genotype required a 40.17% lower dose, and carriers of the CYP2C9*1/*3 genotype required a 35.04% lower dose, compared to wild-type allele carriers for the CYP2C9 gene. Due to the presence of only one patient with dual variant alleles in CYP2C9 (CYP2C9*2/*2 or CYP2C9*2/*3), the effect of these genotypes could not be determined.

For the VKORC1 gene, VKORC1*1/*2A carriers required a 29.91% lower dose, while VKORC1*2A/*2A carriers required a 77.21% lower dose compared to wild-type genotype carriers. The effect of carrying polymorphic variants in both CYP2C9 and VKORC1 genes on the acenocoumarol dose was compared to wild-type genotypes (Table 4). Patients with one variant allele in CYP2C9 and two in VKORC1 required significantly lower doses than carriers of other genotype combinations in these genes. Wild-type carriers for both genes had the highest dose requirement (4.66 ± 1.28 mg/day) compared to patients with different combinations of polymorphic variants.

Single-variable and multiple linear regression methods were applied to assess the genetic and non-genetic factors and their impact on the daily dose of acenocoumarol in the test group of patients undergoing treatment with the coumarin anticoagulant acenocoumarol. The results of the single-variable analysis are presented in Table 5.

Table 3. The effect of relevant genotypes for studied polymorphic variants on the average daily maintenance dose of acenocoumarol in Bulgarian patients with increased thrombosis risk

Gene	Polymorphism	Genotype	N (%)	Average dose ± SD	% reduction/increase in dose against wild type genotype
CYP2C9		*1/*1	76 (64.41)	3.51 ± 2.00	-
	rs1799853	*1/*2	25 (21.19)	2.10 ± 1.40	-40.17
	rs1057910	*1/*3	16 (13.56)	2.28 ± 1.90	-35.04
		*2/*3	1 (0.85)	1.00 ± 0.00	-71.51
HWE	rs1799853 **χ^2 = 1.81** **p = 0.18**	rs1057910 **χ^2 = 0.71** **p = 0.39**	**118**		
APOE		*1/*1	87 (79.09)	3.05 ± 2.02	-
	rs429358	*1/*4	23 (20.91)	2.66 ± 1.76	-11.11
		*4/*4	0 (0.00)	0 ± 0.00	-
HWE	**χ^2 = 1.5** **p = 0.22**		**110**		
APOE	rs7412	*1/*1	99 (90.83)	2.92 ± 1.94	-
		*1/*2	10 (9.17)	3.53 ± 2.28	17.38
HWE	**χ^2 = 0.25** **p = 0.62**		**109**		
VKORC1*2A		*1/*1	48 (40.68)	4.01 ± 1.89	-
	rs9934438	*1/*2A	44 (37.29)	2.96 ± 1.83	-29.91
		*2A/*2A	26 (22.03)	1.30 ± 0.84	-77.21
HWE	**χ^2 = 6.1** **p = 0.01**		**118**		
VKORC1*2B		*1/*1	21 (18.26)	2.14 ± 1.68	-
	rs2884737	*1/*2B	37 (32.17)	2.29 ± 1.74	4.27
		*2B/*2B	57 (49.57)	3.72 ± 1.91	45.01
HWE	**χ^2 = 9.45** **p = 0.002**		**115**		
VKORC1*3		*1/*1	54 (46.96)	2.28 ± 1.95	-
	rs7294	*1/*3	48 (41.74)	3.41 ± 1.61	32.19
		*3/*3	13 (11.30)	4.15 ± 2.16	53.28
HWE	**χ^2 = 0.22** **p = 0.64**		**115**		
VKORC1*4		*1/*1	70 (60.87)	2.69 ± 1.87	-
	rs17708472	*1/*4	39 (33.91)	3.12 ± 1.84	12.25
		*4/*4	6 (5.22)	5.20 ± 2.33	71.51
HWE	**χ^2 = 0.03** **p = 0.85**		**115**		

Table 4. The effect of combined CYP2C9 and VKORC1 genotypes on the average daily maintenance dose of acenocoumarol in Bulgarian patients

VKORC1	*CYP2C9*	N (%)	Average dose ± SD	% dose reduction against VKORC1 GG/CYP2C9*1/*1
1/*1	*1/*1	31 (26.27%)	4.66 ± 1.28	-
	*1/*2	12 (10.17%)	2.61 ± 1.43	-43.99
	*1/*3	4 (3.39%)	2.98 ± 1.43	-36.05
	*2/*3	0 (0.00%)	-	-
1/*2A	*1/*1	33 (27.97%)	3.27 ± 1.73	-29.83
	*1/*2	6 (5.08%)	1.43 ± 1.15	-69.31
	*1/*3	6 (5.08%)	3.07 ± 2.32	-34.12
	*2/*3	0 (0.00%)	-	-
2A/*2A	*1/*1	14 (11.86%)	1.50 ± 0.86	-67.81
	*1/*2	5 (4.24%)	1.15 ± 0.49	-75.32
	*1/*3	6 (5.08%)	1.10 ± 1.08	-78.33
	*2/*3	1 (0.85*)	1.00 ± 0.00	-78.54

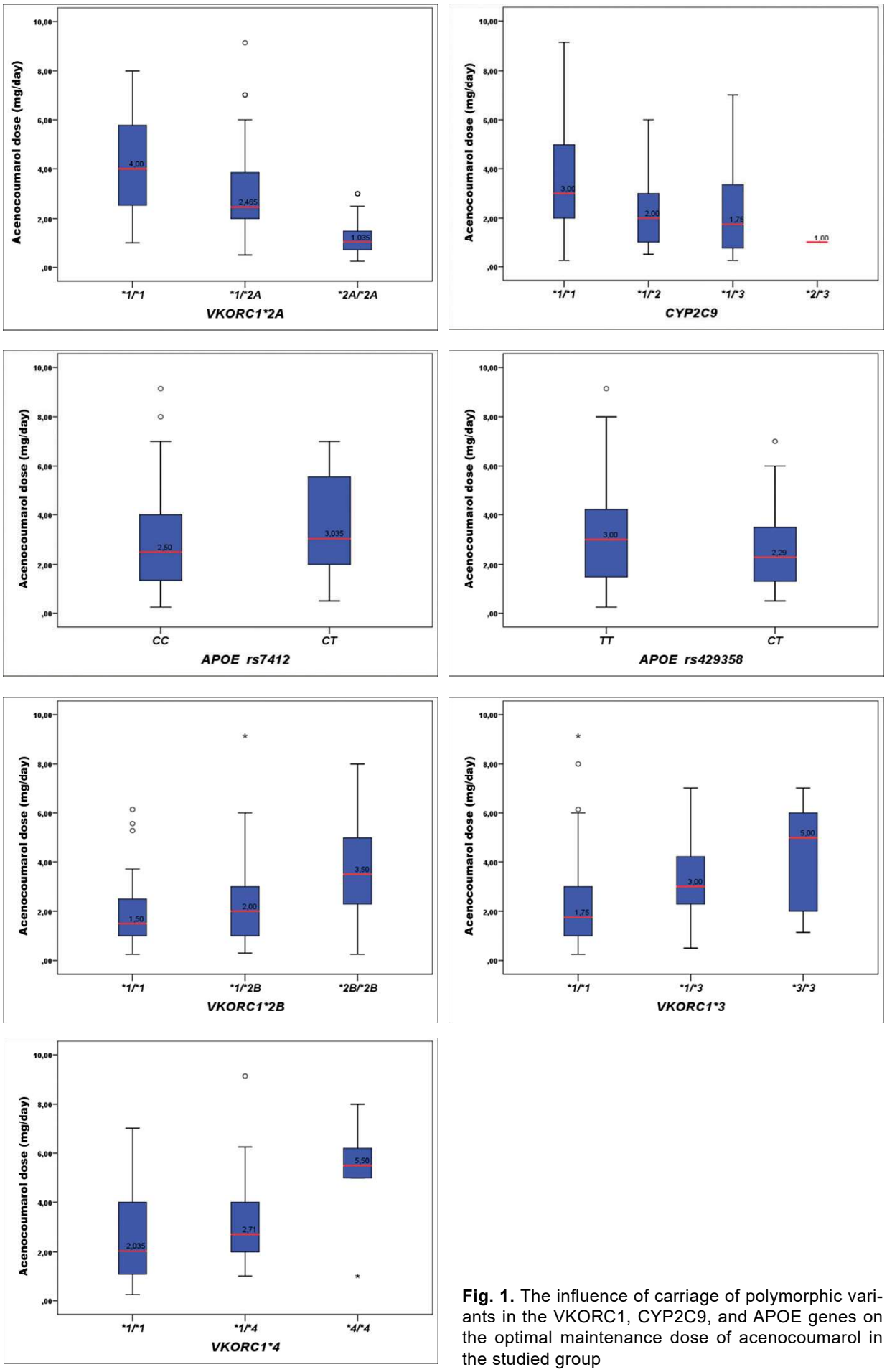

Fig. 1. The influence of carriage of polymorphic variants in the VKORC1, CYP2C9, and APOE genes on the optimal maintenance dose of acenocoumarol in the studied group

Table 5. Genetic and clinical factors influencing the daily optimal maintenance dose of acenocoumarol in the studied group of patients with cardiovascular diseases. R^2 denotes the percentage variability in the indirect anticoagulant dose due to the relevant factor

Factors:	R^2 (%)	Adjusted R^2 (%)	P value
Age	6.4	5.6	0.006
Gender	0.4	< 0.1	0.47
BMI	3.3	1.6	0.17
Height	1.9	0.2	0.30
Weight	1.6	0.5	0.22
Cigarette smoking	< 0.1	< 0.1	0.91
Amiodaron	1.1	0.1	0.29
Statins	2.5	1.4	0.15
Diagnosis	1.8	< 0.1	0.16
VKORC1*2A	26.8	26.1	< 0.001
VKORC1*2B	12.4	11.7	< 0.001
VKORC1*3	12.2	11.4	< 0.001
VKORC1*4	6.1	5.3	0.008
CYP2C9*2	7.2	6.4	0.003
CYP2C9*3	2.9	2.1	0.063
APOE rs429358	0.7	< 0.1	0.40
APOE rs7412	0.8	< 0.1	0.35

To create a pharmacogenetic algorithm, variables such as age, gender, diagnosis, weight, amiodarone and statin intake, and CYP2C9, CYP4F2, APOE, and VKORC1 genotypes were included simultaneously in the multivariate analysis. The β-coefficients represent the relative strength of each independent variable's influence on medication dosing within the model. R^2 indicates the percentage of variability in the dose explained by the relevant factors or model. The reliability of the determined parameters was evaluated by performing a ten-fold cross-validation, where patients were randomly allocated to the main and validation groups. Multivariate analysis revealed that age and the presence of CYP2C9*2, CYP2C9*3, VKORC1*2A, and VKORC1*3 alleles accounted for 43.8% of the variability in the average daily maintenance dose of acenocoumarol (Table 6).

Table 6. Independent genetic and clinical factors influencing the daily optimal maintenance dose of acenocoumarol after application of multiple regression analysis

Independent variables	Unstandardized coefficients		t	P value
	B.	Standard error		
Constant	5.939	0.685	8.664	4.97*10-14
Age (years)	-0.033	0.010	-3.186	0.002
VKORC1*2A	-1.149	0.194	-5.931	3.67*10-8
VKORC1*3	0.433	0.220	1.972	0.05
CYP2C9*2	-1.425	0.334	-4.265	4.30*10-5
CYP2C9*3	-0.486	0.400	-1.213	0.228

The created mathematical algorithm is given in the box below:

***Optimal daily maintenance dose of acenocoumarol** = 5.939 – 0.033*(age in years) – 1.149* (number of VKORC1*2A alleles) + 0.433*(number of VKORC1*3 alleles) – 1.425*(number of CYP2C9*2 alleles) – 0.486*(number of CYP2C9*3 alleles)*

DISCUSSION

Vitamin K antagonists, including acenocoumarol, are commonly used for the treatment and prevention of venous and arterial thrombosis. These drugs have a narrow therapeutic index and exhibit considerable interindividual pharmacokinetic and pharmacodynamic variability. The required dose of anticoagulant to achieve a therapeutic prothrombin time (INR) level can vary significantly, not only between individuals but also within an individual under different conditions. Several factors contribute to this variability, including age, sex, body weight, diet, comorbidities, and genetic factors [12].

One approach to reducing the time required to reach optimal INR values in indirect anticoagulant therapy – thereby enhancing both the efficacy and safety of treatment with these medications – is the use of dosing algorithms that incorporate demographic, clinical, and genetic variables. However, these algorithms need to be validated through randomized clinical trials to assess their effectiveness and potential integration into standard clinical practice.

Recent retrospective studies have shown that polymorphic variants in the CYP2C9 and VKORC1 genes account for approximately one-third of the variability in the dose of indirect anticoagulants. It has been suggested that genetic information could be used to predict the initial and/or maintenance dose of coumarin derivatives in patients at increased risk of thrombosis. While several small randomized controlled trials have explored this approach, there is still no definitive evidence regarding the effectiveness of pharmacogenetic-guided dosing of acenocoumarol and warfarin to improve treatment safety and efficacy [13]. Moreover, the economic advantages of using pharmacogenetic algorithms for anticoagulant dosing remain unclear.

In our study, we aimed to investigate the underlying causes of variability in acenocoumarol response by analyzing selected variants in genes associated with the pharmacokinetics and pharmacodynamics of the medication. This is the first study to examine the relationship between the presence of polymorphic alleles in the VKORC1 and APOE genes and the sensitivity of Bulgarian patients to indirect anticoagulant thera-

py. The study included patients from various disease categories who required long-term oral anticoagulation therapy.

The primary objective of our study was to determine how different clinical factors, as well as polymorphic variants in the CYP2C9, VKORC1, and APOE genes, influence the daily maintenance dose of acenocoumarol in Bulgarian patients with various indications for anticoagulant therapy. The impact of polymorphic variants in these genes on the dosing of oral anticoagulants like warfarin, acenocoumarol, and phenprocoumon has been studied in different ethnic groups to date [9, 10, 14-20].

Our analyses revealed that the allelic and genotypic frequencies for CYP2C9*2 and CYP2C9*3 polymorphisms were consistent with the results of a previous study conducted in Bulgaria [21]. Regarding the drug dose, carriers of the reference genotype for CYP2C9 and VKORC1 required a higher dose of acenocoumarol compared to patients with heterozygous or homozygous alternative alleles.

To date, two polymorphic variants in the VKORC1 gene -1639G>A in the promoter region and 1173C>T in intron 1 – have been associated with a significantly reduced anticoagulant dose requirement. These two polymorphisms are in complete linkage disequilibrium, defining the haplotypes VKORC1*1 (reference) and VKORC1*2A (alternative). The presence of a polymorphic allele for these variants is linked to a lower optimal dose of anticoagulant needed to achieve target INR values between 2 and 4. Over recent years, this relationship has been consistently confirmed across diverse populations, although variability in the influence of VKORC1 polymorphisms on anticoagulant dosing has been observed across studies [8-10, 14, 19, 22-25].

The CYP2C9 gene encodes an enzyme responsible for the oxidative metabolism of several drugs, including warfarin, acenocoumarol, phenprocoumon, and phenytoin (http://medicine.iupui.edu/flockhart/table.htm). Considerable inter-individual variability in enzyme expression and catalytic activity arises from functionally significant genetic variants in CYP2C9. To date, approximately 30 haplotypes have been identified, each formed by individual or combined polymorphic substitutions. The two most extensively studied variants are CYP2C9*2 (430C>T, resulting in the amino acid substitution Arg144Cys) and CYP2C9*3 (1075A>C, resulting in Ile359Leu). These alleles encode enzymes with significantly reduced activity, retaining approximately 12% (CYP2C9*2) and 5% (CYP2C9*3) of the activity of the reference allele CYP2C9*1 [21, 26]. However, these variants appear to have a lesser effect on the optimal maintenance dose of acenocoumarol compared to VKORC1*2A, which accounts for the greatest proportion of observed dose variation [4, 10]. Additionally, genetic variants in CYP3A5 and CYP2C19 have been studied for their role in individual sensitivity to anticoagulant therapy [38, 48].

Age was also found to be significantly associated with the acenocoumarol dose in our study, accounting for 6.4% of the dose variation and leading to a decrease in optimal dose by 0.033 mg/day for each additional year of age. Consequently, older patients require substantially lower doses of acenocoumarol than younger individuals. This association between age and acenocoumarol dose has been observed in other studies as well, although the underlying mechanism remains unclear [14, 17, 27, 28]. Some research suggests that CYP2C9 enzyme activity does not decline with age, while other studies report the opposite [4, 29]. Although renal function generally decreases with age, one study indicated that only patients with end-stage renal disease showed reduced CYP2C activity [30]. Additionally, older adults on low-vitamin K diets may experience a deficiency in this vitamin, which can reduce the levels of vitamin K-dependent clotting factors [2].

Body mass index (BMI) is another clinical factor potentially influencing acenocoumarol dosing. In our study, a direct relationship was found between BMI and optimal medication dose, though this association was not statistically significant in multivariate analysis. Consequently, BMI's effect on dose variability was considered non-significant. However, BMI was identified as a significant predictor of the optimal acenocoumarol dose in dosing algorithms developed by Borobia et al. [10] and Van Schie et al. [4], which contrasts with our findings.

Gender has also been reported as a variable influencing acenocoumarol dosing in several studies, and it is included in the EU-PACT survey algorithm [4, 31]. In our study, however, we found no difference in therapeutic dosing between men and women, consistent with other studies that similarly did not find gender to impact the optimal dose for achieving a stable INR [4, 10, 31].

Our study found no significant difference in the maintenance dose of anticoagulants among patients taking concurrent medications that impact the metabolism of vitamin K antagonists, such as amiodarone and statins. In the EU-PACT algorithm, amiodarone intake had the lowest predictive value among all factors (R^2 = 0.2%) [4, 10, 31]. Van Schie et al. reported a slight reduction in the mean daily dose of aceno-

coumarol in patients using statins: 0.11 mg less for those on atorvastatin and 0.29 mg less for those on simvastatin [4, 10, 31]. In contrast, the EU-PACT algorithm excluded statins, likely due to their minimal impact on acenocoumarol dosing [4].

In addition to CYP2C9 and VKORC1 polymorphisms, several studies have identified polymorphic variants in the CYP4F2 gene as important predictors of acenocoumarol maintenance dose, accounting for 1-2% of dose variability [32]. This factor has been included in previous dosing algorithms [5, 32, 33]. A genome-wide study by Teichert et al. [34, 35] indicated that genetic polymorphisms in CYP2C9, VKORC1, CYP4F2, and CYP2C18, alongside clinical variables like age, gender, BMI, and target INR, together explained 48.8% of the variability in acenocoumarol dosing. However, CYP2C18 contributed only 1.2% of this variability and was not deemed significant. Additional studies suggest that CYP2C18 may be associated with a lower dose requirement for anticoagulants [36, 37].

In Bulgaria, only one study has examined the relationship between genetic variations and the dosing of the oral anticoagulant acenocoumarol [21, 26]. Saraeva et al. investigated genes involved in the metabolism and transport of acenocoumarol (CYP2C9, CYP2C19, CYP1A2, CYP3A4, CYP3A5, and ABCB1) in a Bulgarian cohort. This study found that the CYP2C9*2, CYP2C9*3, and ABCB1 2677GG/3435CC haplotypes were associated with significantly lower dose requirements of acenocoumarol. Unlike our study, however, Saraeva et al. did not assess polymorphic variants in the VKORC1 gene and categorized patients based on their acenocoumarol dose. Our objective was not only to establish an association between specific genetic variants and dose requirements but also to develop a pharmacogenetic model for predicting the optimal dose based on clinical and genetic factors.

Numerous studies have developed pharmacogenetic algorithms to predict the initial dose of acenocoumarol [4, 5, 8-10, 14-16, 38, 39]. These studies employed univariate and multivariate linear regression analyses to evaluate the impact of genetic and non-genetic factors on the optimal daily maintenance dose. Variables such as age, gender, diagnosis, weight, amiodarone use, and genetic variants in CYP2C9, CYP4F2, GCCX, APOE, and VKORC1-1639G>A were included in the final regression models to create the most accurate pharmacogenetic algorithms.

Existing dosing algorithms are typically population-specific, meaning that ready-made algorithms cannot simply be applied to the Bulgarian population. Our study focuses on developing an algorithm specifically tailored to predict therapeutic doses of acenocoumarol for Bulgarian patients, based on clinical and genetic data. To create this therapeutic algorithm, we first identified key factors influencing dose requirements. In our multivariate analysis, we included age as a clinical and demographic factor, alongside polymorphic variants in the VKORC1 and CYP2C9 genes as genetic factors affecting the therapeutic dose of acenocoumarol. Our findings suggest that acenocoumarol dosing based on a pharmacogenetic algorithm could potentially reduce adverse effects of anticoagulant therapy, such as bleeding and thrombosis.

Similar to other studies, our algorithm development involved univariate and multivariate linear regression analyses to evaluate the impact of genetic and non-genetic factors on the optimal daily maintenance dose of acenocoumarol. Variables such as age, gender, diagnosis, weight, amiodarone use, and genetic variants in CYP2C9, CYP4F2, GCCX, APOE, and VKORC1-1639G>A were included in these analyses to construct the most accurate pharmacogenetic models.

The model we developed explained 43.8% of the variability in acenocoumarol dose based on data from the test group, which was somewhat lower than that reported by Van Schie et al. (52.6%) [4] and Borobia et al. (60.6%) [10]. This difference can be attributed to the inclusion of additional polymorphic variants in the CYP4F2 and APOE genes in Borobia et al.'s algorithm, which together accounted for about 5% of the observed dose variability.

CONCLUSION

In conclusion, polymorphic variants in the CYP2C9 and VKORC1 genes (CYP2C9*2, CYP2C9*3, VKORC1*2A, and VKORC1*3) significantly influence the average daily maintenance dose of coumarin anticoagulants in Bulgarians. These findings align with results reported for other populations of European and Asian origin [4-6, 10, 14, 15, 24, 29]. Based on this study's findings, we developed a pharmacogenetic algorithm tailored to predict the optimal starting and maintenance dose of acenocoumarol for Bulgarian patients at elevated risk of thrombosis who require anticoagulation. Further research incorporating additional genetic factors, such as polymorphisms in CYP4F2, CYP2C18, GGCX, and EPHX1, in a larger patient cohort could significantly enhance the accuracy of this dosing model.

Ethical standards: *All procedures involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committees (Ethics Committees of the Medical University – Sofia and the Medical University of Pleven) and in line with the 1964 Helsinki Declaration and its subsequent amendments or comparable ethical guidelines.)*

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

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