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Genotype-phenotype correlations in patients treated with acenocoumarol

Corelaţii genotip-fenotip la pacienţii trataţi cu acenocumarol

Florentina Claudia Militaru, Ștefan Cristian Vesa*, Sorin Crișan, Valentin Militaru, Adrian Pavel Trifa, Anca Dana Buzoianu

"Iuliu Haţieganu" University of Medicine and Pharmacy, România

Abstract

***Aim**: This research aims to establish a genotype-phenotype correlation in patients treated with acenocoumarol and studies the genetic factors (VKORC1 and CYP2C9 polymorphisms) that may influence INR values during initiation of oral anticoagulant therapy with acenocoumarol. **Material and methods**: We included 131 patients that needed treatment with acenocoumarol, 70 (53.4%) women and 61 (46.6%) men, observed at the 5th Medical Clinic in Cluj-Napoca, between 2009-2011. We studied the influence of age, gender, concomitant medication and of CYP2C9 and VKORC1 genes on the INR value recorded on the third day of treatment and on the difference between this value and the initial INR value at the starting point for the treatment (INRDIF). **Results and conclusion**: We demonstrated a statistically significant difference for INR3 and INRDIF values in patients with AA genotype and those with GG genotype of the c.-1639G>A polymorphism of the VKORC1 gene. The presence of AA genotype of the c.-1639G>A polymorphism of the VKORC1 gene determined a 15.7-fold increase in the risk that a patient might display supratherapeutic INR after 2 days of treatment with 4 mg of acenocoumarol.*

***Keywords**: Acenocoumarol, CYP2C9, INR, VKORC1*

Rezumat

***Scop:** Această cercetare are drept scop stabilirea unei corelații genotip-fenotip la pacienții trataţi cu acenocumarol şi studierea factoriilor genetici (polimorfismele VKORC1 și CYP2C9), care ar putea influența valorile INR în timpul inițierii terapiei anticoagulante orale cu acenocumarol. **Material și metode**: Am inclus 131 de pacienți care necesitau tratament cu acenocumarol, 70 (53,4%) femei și 61 (46,6%) bărbați, internaţi în Clinica Medicala 5 din Cluj-Napoca, între 2009-2011. Am studiat influența vârstei, sexului, medicației concomitente și a genelor CYP2C9 și VKORC1 asupra valorii INR măsurate în ziua a treia de tratament şi asupra diferenţei dintre această valoare şi valoarea INR măsurată la debutul tratamentului (INRDIF). **Rezultate și concluzii**: Am demonstrat o diferență semnificativă statistic pentru INR3 și valorile INRDIF la pacienții cu genotip AA și cei cu genotip GG ai polimorfismului c.- 1639G>A al genei VKORC1. Prezența genotipului AA al polimorfismului c.- 1639G>A al genei VKORC1 a determinat o creștere de 15,7 ori a riscului ca un pacient să prezinte INR supraterapeutic după 2 zile de tratament cu 4 mg de acenocumarol.*

***Corresponding author**: Ștefan Cristian Vesa, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, România e-mail: stefanvesa@gmail.com

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Introduction

Acenocoumarol, a coumarin derivative oral anticoagulant, is used in Romania as it is in Europe, with indications for prophylaxis and treatment of thromboembolic events. For practitioners, prescribing a treatment with acenocoumarol is a challenge, requiring close monitoring by a systematic identification of International Normalised Ratio (INR) values. It is known that oral anticoagulants show a significant variability in treatment response. The response to treatment with acenocoumarol is assessed by INR, and INR values between 2 and 3 are considered to ensure therapeutic efficacy with low bleeding risk. This target is sometimes difficult to achieve due to a series of factors that may influence response to treatment and INR values. Studies on patients treated with oral anticoagulants showed that response to treatment is influenced by demographic factors - age, gender, diet, race, as well as genetic factors, as shown by pharmacogenetic research (1-4). In this last category of factors, the most important role is played by the genes encoding cytochrome P4502C9 (*CYP2C9*) enzymes and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) (5).

Demographic and environmental factors have long been regarded as the main factors responsible for inter- and intra-individual variations in the response to oral anticoagulant treatment. Among these factors are: patient characteristics (age, gender, body mass index), dietary intake of vitamin K, comorbidities (acute liver failure, kidney failure, heart failure, thyroid diseases, etc.), acute intercurrent pathologies (fever, sepsis, acute decompensated heart failure, diarrhea, etc.), and concomitant medication (6, 7).

Cytochrome P4502C9 (*CYP2C9*) is the key enzyme in the hepatic metabolism of acenocoumarol. Pharmacogenetic studies have shown that *CYP2C9* is a polymorphic enzyme due to its genetic variability, which in turn is responsible for fluctuations in enzymatic activity (1, 4, 8).CYP2C9 polymorphisms vary according to race (Caucasian, African or Asian).

In Caucasians, 8-19% of individuals carry at least one *CYP2C9*2* (Arg 144-Cys) allele and 6-10% of individuals carry at least one *CYP2C9*3* (Ile 359-Leu) allele. Therefore, about a quarter of the general Caucasian population carry at least one variant allele. Variant enzymes resulting from these polymorphisms are less active than normal enzymes, decreasing the metabolism of coumarin derivatives: subjects carrying at least one mutant allele have an increased sensitivity to these derivatives and to an associated bleeding risk, being called "poor metabolizers" (9).

Twenty-eight polymorphisms have been described for the *VKORC1* gene, most of them being placed in three major groups, which account for most of the genetic variability of *VKORC1*. The three major VKORC haplotypes (VKORC1*2, *3 and *4) are defined by the following tagging SNPs: G3673A (rs9923231); C6484T (rs9934438) and G9041A (rs7294). The A and T alleles of the G3673A and C6484T SNPs define the VKORC1*2 haplotype, while the G and A alleles of the G9041A SNP define the VKORC1*4 and VKORC*3 haplotypes, respectively. VKORC1*1 denotes the reference haplotype, that is the reference sequence of the VKORC1 gene. *VKORC1*2* haplotype was found responsible for most of the variations in response to oral anticoagulants (10). Montes et al have also shown that the A allele of the *VKORC1* gene's c.-1639G>A polymorphism, which tags the *VKORC1*2* haplotype, is associated with the

need for a lower dose of acenocoumarol in patients with anticoagulant therapy (11).

This research aims to establish a genotype-phenotype correlation in patients treated with acenocoumarol and studies the genetic factors that may influence INR values during initiation of oral anticoagulant therapy with acenocoumarol. We aimed to study the influence of demographic and genetic factors on INR values at the beginning of oral anticoagulant therapy with acenocoumarol, INR representing the phenotypic expression of the patients' enzyme genotype (*VKORC1* and *CYP2C9*).

In order to achieve this, we studied the influence of age, gender, concomitant medication and of *CYP2C9* and *VKORC1* genes on the INR value recorded on the third day of treatment and on the difference between this value and the initial INR value at the starting point for the treatment (INRDIF).

Material and method

We included 131 patients treated with acenocoumarol, 70 (53.4%) women and 61 (46.6%) men, observed at the 5th Medical Clinic in Cluj-Napoca, between 2009-2011. The study protocol was approved by the Ethics Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca. Patients were included in the study after signing their informed consent to their participation in the study and to the genetic determinations.

The following variables were recorded for each patient: age, gender, patient origin (rural-urban area); indications for oral anticoagulant treatment: deep vein thrombosis (DVT), pulmonary embolism, atrial fibrillation (AF); concomitant medication: we recorded the medication that can influence the metabolism of oral anticoagulants of cytochrome P450 enzyme system - statins, proton pump inhibitors and spironolactone; initial INR values (INR0) and INR values on the third day of treatment (INR3) were recorded. All patients in the study underwent treatment with acenocoumarol, with an initial dose of 4 mg in both the first and second day. We considered the 3-day interval as adequate to evaluate the anticoagulant effect of acenocoumarol, knowing that acenocoumarol acts after 18 to 24 hours and peaks at 36-48 hours after starting the treatment.

Blood samples were obtained by venepuncture, using a vacutainer containing natrium citrate 0.109 M. The samples were analysed within two hours from blood collection. The optic method was used for analysis and the time needed for the observation of first fibrin filaments was recorded. The samples were analysed on an automated coagulometer Thrombolyser XT and we used thromboplastin Technoclone HIS batch 6T43B00 with ISI value of 1.11. The calibration of the analyzer was performed with plasma, reference batch 1R01b01.01. For the internal control we used two plasma sets, normal and pathologic, batch 1P44B00 and 3P24B00, respectively. INR reference range was considered between 0.8 and 1.2 by the laboratory.

We genotyped the *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* c.-1693 G>A polymorphisms using PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) assay, essentially as described in references (12, 13).

Briefly, a 372-bp amplicon was obtained by PCR to study the *CYP2C9*2* allele. The amplicon was digested overnight with the Sau96I restriction enzyme (Fermentas MBI, Vilnius, Lithuania), giving rise to 3 fragments with lengths of 179 bp, 119 bp and 74 bp in the case of the wild-type allele. However, the *CYP2C9*2* allele abolishes one restriction site of the Sau96I enzyme, giving rise to only 2 fragments, with lengths of 253 bp and 119 bp. To analyse the *CYP2C9*3* variant, a 130-bp amplicon was obtained by PCR. This amplicon was digested overnight with the StyI restriction enzyme (Fermentas MBI,

Vilnius, Lithuania), and the wild-type allele was resistant to the StyI digestion. However, the *CYP2C9*3* allele creates a restriction site for StyI, cutting the 130-bp amplicon into 2 fragments, with lengths of 104 bp and 26 bp.

In order to study the *VKORC1* c.-1693 G>A polymorphism, a 290-bp amplicon was obtained by PCR. The amplicon was digested overnight with MspI restriction enzyme (Fermentas MBI, Vilnius, Lithuania). If the G allele was present, then the 290-bp amplicon was cut in 2 fragments, with lengths of 167 bp and 123 bp, whereas the presence of the A allele rendered the amplicon resistant to digestion with the MspI restriction enzyme.

All PCR reactions were carried out in Mastercylcer thermocyclers (Eppendorf, Germany).

The restricted PCR products were resolved in 3% MetaPhor agarose gel electrophoresis (Lonza, Rockland, USA), stained with ethidium bromide.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 17, Chicago, IL, USA). Data were labelled as nominal or quantitative variables. Nominal variables were characterized by means of frequencies. Quantitative variables were tested for normality of distribution using Kolmogorov-Smirnov test and were described by mean ± standard deviation or median and interquartile range, whenever appropriate. The frequencies of nominal variables were compared with a chi-square test. Differences in the mean or median between groups were analysed using the t test, Mann–Whitney test, Kruskal–Wallis test or ANOVA, when appropriate. We used Wilcoxon test in order to determine the differences between two repeated measures of a variable.

Multivariate linear regression was used in order to determine the variables with independent influence on the INRDIF, after base-10 logarithmic transformation. The analysis of the influence of the studied parameters on the probability that a patient might display a supratherapeutic (INR3 > 3), subtherapeutic (INR3 < 2) or therapeutic INR3 was accomplished using a multinomial logistic regression.

The level of statistical significance was set at $p<0.05$.

Results

The characteristics of the patient group are summarized in *table I*.

The minimum age was 21 years, the maximum 92 years and the mean 65.7±12.9 years.

Initial INR values (INR0) recorded on admission, before beginning the anticoagulant therapy were: the minimum 0.81, the maximum 1.24, and the mean 1.13 ± 0.16. INR values recorded on the third day of oral anticoagulant treatment (INR3) were: the minimum 0.88, the maximum 5.99, and the median 1.91 (1.19). We determined highly statistical significant differences between

Table I. Patient group characteristics

Variable	Number of patients (%)
Men	61 (46.6%)
Women	70 (53.4%)
Rural area	72 (55.0%)
Urban area	59 (45.0%)
DVT	116 (88.5%)
AF	24 (18.3%)
PPI treatment	13 (9.9%)
Spironolactone treatment	16 (12.2%)
Statins treatment	68 (51.9%)
*CYP2C9*1/CYP2C9*1*	80 (61.1%)
*CYP2C9*1/CYP2C9*2*	24 (18.3%)
*CYP2C9*1/CYP2C9*3*	22 (16.8%)
*CYP2C9*2/CYP2C9*3*	5 (3.8%)
VKORC1-GG	39 (29.8%)
VKORC1-GA	74 (56.5%)
VKORC1-AA	18 (13.7%)

INR0 and INR3 values (p<0.001). We calculated the differences between INR3 and INR0 (INRDIF) values and obtained a minimum value of 0, a maximum of 4.49, and median of 0.76 (1.07).

The differences between several demographic, pharmacologic and genetic factors regarding INR3 can be seen in *table II*.

The differences between several demographic, pharmacologic and genetic factors regarding INRDIF can be seen in *table III*.

To assess the concomitant effect of the studied parameters on INRDIF, we applied a multiple linear regression using INRDIF (log-transformed) values as dependent variables. The only variable that independently influenced INRDIF was the c.-1639G>A polymorphism of the *VKORC1* gene (p=0.001).

In the next stage we divided the group into three categories according to INR3 values: 39 patients with subtherapeutic INR (29.8%), 70 patients with therapeutic INR (53.4%), 22 patients with supratherapeutic INR (16.8%).

Patient age did not differ in the three groups (p=0.92). There have been no significant differences between the groups regarding: patient gender (p=0.66), concomitant medication with proton pump inhibitors (p=0.76), spironolactone (p=0.47), statins (p=0.62), the presence of CYP2C9*2 allele (p=0.97), or the presence of CYP2C9*3 allele (p=0.2).

The share of the genotypes of the *VKORC1* gene's c.-1639G>A polymorphism was different in the three subgroups (p=0.025).

The analysis of the influence of the studied parameters on the probability that a patient might display a supratherapeutic, subtherapeutic or therapeutic INR3 was accomplished by using a multinomial logistic regression. The group of patients with subtherapeutic INR3 was used as reference dependent variable.

Table II. Differences between demographic, pharmacologic and genetic variables regarding INR3 levels

Variable			INR3	P
Men			1.88 (0.98)	0.56
Women			2 (1.38)	
PPI treatment	Yes		2.21 (1.27)	0.23
	No		1.9 (1.23)	
Spironolactone treatment		Yes	1.72 (1.7)	0.75
		No	1.98 (1.14)	
Statins treatment	Yes		1.89 (1.22)	0.47
	No		2 (1.12)	
*CYP2C9*1/CYP2C9*1*			1.89 (1.34)	0.9
*CYP2C9*1/CYP2C9*2*			2 (1.09)	0.62
*CYP2C9*1/CYP2C9*3*			1.99 (1.37)	0.4
*CYP2C9*2/CYP2C9*3*			1.85 (1.2)	0.56
VKORC1-GG			1.7 (0.56)	0.01
VKORC1-GA			2 (1.51)	
VKORC1-AA			2.78 (1.2)	

Table III. Differences between demographic, pharmacologic and genetic variables regarding INRDIF levels

Variable			INR3	P
Men			0.75 (0.88)	0.64
Women			0.81 (1.37)	
PPI treatment	Yes		1.13 (1.27)	0.09
	No		0.8 (0.96)	
Spironolactone treatment		Yes	0.5 (2)	0.75
		No	0.8 (1.14)	
Statins treatment	Yes		0.74 (1.17)	0.47
	No		0.81 (1.12)	
*CYP2C9*1/CYP2C9*1*			0.75 (1.29)	0.8
*CYP2C9*1/CYP2C9*2*			0.78 (0.92)	0.68
*CYP2C9*1/CYP2C9*3*			0.78 (1.18)	0.69
*CYP2C9*2/CYP2C9*3*			0.81 (1.23)	0.7
VKORC1-GG			0.65 (0.38)	0.01
VKORC1-GA			0.85 (1.43)	
VKORC1-AA			1.54 (1.55)	

The presence of AA genotype of the *VKORC1* gene's c.-1639G>A polymorphism determined a 15.7-fold increase in the risk that a patient might display supratherapeutic INR levels after 2 days of treatment with 4 mg of acenocoumarol (p=0.001; CI95% 2.34-105.38). Presence of the GA genotype of the c.-1639G>A polymorphism of the *VKORC1* gene only determined a 4.8-fold increase in the risk of a patient displaying supratherapeutic INR values after 2 days of treatment with 4 mg of acenocoumarol (p=0.04; CI95% 1-23.34).

Discussion

In this study we aimed to assess the influence of demographic and genetic factors on one of the components of the response to treatment with acenocoumarol, namely the INR values. They have direct practical importance because they dictate the therapeutic conduct and the acenocoumarol dose necessary to achieve the targeted therapeutic response with a minimal risk of bleeding.

Demographic factors - age and gender - did not exert any influence on INR3. Spreafico et al conducted a similar study to the one carried out by us, analyzing the impact of certain factors on INR values, except that their study focused on the INR value on day 4 after starting the treatment with acenocoumarol. Similarly to our study, their study found no association between patient gender and INR value. As opposed to our results, their results show a positive association between patient age and INR value (14).

Concomitant medication with proton pump inhibitors, statins or spironolactone had no influence on INR values, according to our results. Our study considered the classes of drugs that are known to influence the pharmacokinetics of oral anticoagulants. The study carried out by Spreafico et al also showed no influence of the concomitant medication on INR values (14). The existence of *CYP2C9*2* and *CYP2C9*3* variants did not influence INR3 values, compared with patients carrying the wild-type *CYP2C9* variant. Verhoef et al have shown that carriers of *CYP2C9*3* allele displayed supratherapeutic INR values during the initiation of the therapy with acenocoumarol, compared with patients who were not carriers of *CYP2C9*3* allele. The authors of the same research found a clinical relevance for the carriers of the *CYP2C9*2* allele (8). Other results, reported by Spreafico, show a higher mean INR determined on the fourth day after starting the treatment with acenocoumarol in patients carrying at least one *CYP2C9*3* allele compared to *CYP2C9*1/*1* patients. The highest INR values were recorded in *CYP2C9*3/*3* homozygous patients (14). Also, *CYP2C9*2* carriers had higher INR values. In the study conducted by Teichert et al, a slight tendency for elevated INR values was recorded among carriers of *CYP2C9*2* or **3* alleles, but without statistical significance (15).

The results obtained in this study showed that there is a statistically significant difference between INR3 values in patients with AA genotype and those with GG genotype of the c.-1639G>A polymorphism of the *VKORC1* gene. Patients carrying the A allele had more elevated INR3 values. Most results published in the literature show that the presence of *VKORC1* polymorphisms influences INR values. Bodin et al showed that subjects with the c.-1639G>A polymorphism of the *VKORC1* gene have higher INR values (5). Also, the results reported by Spreafico show that patients who had INR values >6 on day 4 after starting the treatment were *VKORC1 *2/*2* homozygous patients, which is in agreement with the results obtained by our study (14).

Unlike our study and those conducted by Bodin and Spreafico, the results reported by Schalekamp show that only the co-existence of *VKORC1* and *CYP2C9* polymorphisms in a pa-

tient may lead to an INR>6 compared to wild-type patients or carriers of a single *CYP2C9* or *VKORC1* polymorphism (16).

Limdi et al showed that, in patients treated with warfarin, the presence of a *VKORC1* variant or the co-existence of *CYP2C9* + *VKORC1* polymorphisms increases the risk of supratherapeutic INR, while *CYP2C9* polymorphisms don't have this influence (17).

Our results on patients treated with acenocoumarol are similar, except that they did not show a statistical significance for those who associate *CYP2C9* and *VKORC1* polymorphisms.

In an attempt to establish a genotype-phenotype correlation, we defined a parameter called INRDIF reflecting the difference between INR3 and INR1 values. We considered that this parameter can reflect response variation to treatment, being in close contact with INR values after starting the treatment with acenocoumarol. This is also demonstrated by the statistical analysis of the results obtained. There was no statistically significant association between age, gender, concomitant medication, *CYP2C9*2* or **3* alleles and INRDIF or INR3. Instead, we demonstrated the existence of a statistically significant difference between INRDIF or INR3 values in patients with AA genotype and in those with GA genotype of the c.-1639G>A polymorphism of the *VKORC1*2* gene. Patients with AA genotype had a higher INRDIF value. We did not find a definition of this parameter in the literature, so there could be no comparisons. Instead, Limdi studied INR growth rate/day at the starting point for the treatment with warfarin, showing that this parameter is influenced by *CYP2C9* and *VKORC1* genotype (17).

We found that the presence of AA genotype of the c.-1639G>A polymorphism of the *VKORC1* gene increased by a 15.7-fold the risk that a patient might display a supratherapeutic INR after 2 days of treatment with 4 mg of acenocoumarol. Thus, our study is consistent with the results published in the literature showing that *VKORC1* polymorphisms increase the risk that a patient might display INR values above the therapeutic range (5, 17, 18).

Our study had several limitations: we could not measure the impact of diet; we did not determine the genotype for the polymorphisms associated with resistance to acenocoumarol.

The c.-1639G>A polymorphism of the *VKORC1* gene had an important influence on the phenotypic expression of treatment with acenocoumarol. The *CYP2C9* polymorphisms have not determined a significant phenotypic response.

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The authors declare that they have no conflict of interest.

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