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Effect of *CYP2C9*, *VKORC1*, *CYP4F2*, and *GGCX* gene variants and patient characteristics on acenocoumarol maintenance dose: Proposal for a dosing algorithm for Moroccan patients

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Summary **We investigated the impact of non-genetics factors, and single nucleotide polymorphisms (SNPs) in *VKORC1*, *CYP2C9*, *CYP4F2*, and *GGCX* on acenocoumarol dosage in Moroccan adult's patients, in order to develop an algorithm to predict acenocoumarol dose for Moroccan patients. Our study consisted of 217 Moroccan patients taking a maintenance dose of acenocoumarol for various indications. The patients were genotyped for *VKORC1* –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9*2*, *CYP2C9*3*, *CYP4F2* 1347 G>A and *GGCX* 12970 C>G SNPs. The statistical analysis was performed using the SPSS software. The age and SNPs in *VKORC1* and *CYP2C9* were significantly associated with the weekly acenocoumarol dose requirement ($p = 0.023$, $p = 0.0001$ and $p = 0.001$ respectively). There was no association found between the weekly acenocoumarol dose and the *CYP4F2* or *GGCX* variants (p-value > 0.05). Non-parametric analysis confirmed the accumulate effect of variant alleles at *VKORC1* –1639 G>A, *VKORC1* 1173 C>T and *CYP2C9* SNPs on the acenocoumarol dose requirement. With 90.24% less dose required for one patient carrying homozygote variant at *VKORC1* –1173 (TT) and *CYP2C9* *x/*x haplotype. The multiple linear regression analysis showed that mutation in *VKORC1* –1639, *VKORC1* 1173 SNPs, or in *CYP2C9* haplotype reduces the mean acenocoumarol weekly dose to 25.4%, 23.4% and 6.2%, respectively. The R2 for multiple regression analysis final model was found to be 35.9%. In this work we were able to establish the factors influencing interindividual sensitivity to the anticoagulant therapy that can help physicians to predict optimal dose requirement for long term therapy.**

Keywords: Acenocoumarol, genetics and non genetics factors, algorithm dose, Morocco

1. Introduction

Acenocoumarol and warfarin are oral anticoagulants of the family of vitamin K antagonists. They are the most prescribed in the prevention and treatment of arterial and venous thromboembolic illnesses (*1*). Interindividual variation in response to therapy constitutes a serious problem for determining the appropriate vitamin K antagonists dose (*2*). This variability is known to depend on many environmental and non-genetic factors; nevertheless a genetic factor has also been reported to play an important role in the variability of the appropriate dose and antivitamin K drug metabolism (*3-7*).

Two genes identified as cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) are mostly described to influence the pharmacokinetic and pharmacodynamic parameters

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of vitamin K antagonists (*6,8-11*). CYP2C9 is the main enzyme responsible for the biotransformation and subsequent elimination of vitamin K antagonists (*11,12*). Available data indicate that the *CYP2C9* is polymorphic (3 major alleles: *CYP2C9*1* wild-type allele, *CYP2C9*2* and *CYP2C9*3* variant alleles), and its genetic variability has been reported to be related with variations in the levels of enzyme activity (*12-14*). The two defective alleles *CYP2C9*2* and *CYP2C9*3* were characterized as slow metabolizers and known to be strongly associated with dose requirement and bleeding complications of vitamin K antagonist (*12-17*). *VKORC1* code for the target enzyme of coumarins (*18-20*). Vitamin K antagonists exert its effect through inhibition of the *VKORC1* gene product. Genetic variations within *VKORC1* result in changed sensitivity to vitamin K antagonists. Available data indicate that the single nucleotide polymorphism (SNP) in the promoter region at the nucleotide position –1639 G>A for *VKORC1* gene and there intronic polymorphism 1173 C>T were found to be strongly affecting vitamin K antagonists dosage (*21*). These SNPs were found to be in linkage disequilibrium (*22*). Patients carrying the wild type G allele for the *VKORC1* –1639 G>A required a higher dose of vitamin K antagonists compared with those carrying the A variant allele (*23*). Also, patients carrying the wild type C allele for *VKORC1* 1173 C>T required a higher dose of vitamin K antagonists compared with those carrying the variant T allele (*24*).

Recently, the genes of cytochrome P450 4F2 (*CYP4F2*) and gamma glutamyl carboxylase (*GGCX*) also contributed moderately to the variability of vitamin K antagonists dose estimated between 1-2% in Caucasian patients (*25,26*). *CYP4F2* gene encodes an enzyme that metabolizes vitamin K1 to hydroxyvitamin K1 (*25*). Whereas GGCX is the enzyme responsible for gamma carboxylation of vitamin K-dependent proteins (*27*).

Acenocoumarol is mostly prescribed in Europe and Africa. Many works have been undertaken to study the implication of the genetic polymorphism of the *CYP2C9* and *VKORC1* on inter-individual acenocoumarol dose variability. In Morocco, acenocoumarol is the only one vitamin K antagonist drug that has had its marketing authorization. However, only a few small studies have investigated the contribution of the non-genetic factors, *CYP4F2*, *CYP2C9* and *VKORC1* –1639 G>A polymorphisms on acenocoumarol dose requirements (*28,29*). And little information is available on the possible implication of *VKORC1* 1173 or *GGCX* SNPs on the subject in our country. The aim of this study was to evaluate the influence of non-genetic and *CYP2C9*, *VKORC1* –1639, *VKORC1* 1173, *CYP4F2* and *GGCX* polymorphisms on acenocoumarol maintenance dose in a cohort of Moroccan adult patients, in order to develop a useful algorithm for predicting anticoagulant dose for Moroccan patients.

2. Materials and Methods

2.1. *Ethic statement*

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the Faculty of Medicine and Pharmacy of Rabat, Mohamed V University, Morocco following the guidelines set by the Declaration of Helsinki. For each patient, an information sheet has been completed with epidemiological data. Written informed consent was obtained from all patients after being informed of the purpose of our work.

2.2. *Patients and sample collection*

A total of 217 patients from different regions of Morocco were recruited in this study. They were registered between January 2015 to December 2016 in the various clinical departments of the Mohamed V Military Teaching Hospital (MVMTH) at Rabat for the surveillance of Thromboembolic (TE) disease, atrial fibrillation (AF) or heart valves. Patients were 17 years or older and were taking a maintenance dose of acenocoumarol to maintain an International Normalized Ratio (INR) of 2.0-3.0.

Cases were defined as those whose acenocoumarol dose requirement has retained constant for at least four successive clinic visits. All these patients are referred to the laboratory of Hematology in the MVMTH for the realization of the prothrombin time (PT)/INR analysis.
Upon arrival at the Laboratory, a blood sample was collected (4 mL) into a sterile EDTA vacutainer and stored at 4°C until further processing for DNA extraction, and a sample was collected in a sterile citrate vacutainer for INR assessment. Both samples were collected in the same visit; the INR sample was processed on the same day while the DNA samples were referred for genetic testing in the Laboratory of Medical Biotechnology, Faculty of Medicine and Pharmacy of Rabat.

Patients who were on concomitant therapy with drugs potentially interacting with acenocoumarol, patients with abnormal blood tests of renal or hepatic function; lacting women and alcoholics were excluded from the study.

2.3. *DNA extraction*

DNA was extracted from blood samples using the QIAamp DNA Blood Extraction Kit according to the manufacturer's instructions (Qiagen, Germany). DNA was quantified by NanoDrop Analyser (ND 1000) spectrophotometer (NanoDrop Technologies, Wilmington, USA). The ratio of absorbance at 260 and 280 nm of DNA was between 1.7 and 1.9.

2.4. *Amplification and purification*

Amplification reaction of each *VKORC1* –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9*2*, *CYP2C9*3*, *CYP4F2* 1347 G>A and *GGCX* 12970 C>G SNPs was carried

out using the Master Mix 2X (Bioline, London, UK), 400 nM of each appropriate primers (Eurogentec, Belgium) (Table 1), and 60 ng of template DNA in a 25 µL reaction volume under the following conditions: preheating at 95°C for 3 min, then 35 cycles of (95°C, 30 s; 58°C, 30 s; 72°C, 30 s), followed by final extension of 5 min at 72°C employing a thermal cycler. The PCR amplicons of *VKORC1*, *CYP2C9*, and *CYP4F2* gene were electrophoresed on a 3% agarose gel and on a 1% agarose gel for *GGCX* gene.

2.5. *Genotyping*

After the PCR reaction, the amplification products were purified using ISOLATE II PCR and Gel Kit according to the manufacturer's instructions (Bioline, London, UK). The purified amplicons were genotyped for all investigated SNPs using Restriction Fragment length Plymorphism (RFLP) technique. So, the purified amplicons was digested with 2 units of restriction enzyme (Biolabs, New England) overnight at 37°C. The digested products were analyzed on 3% agarose gel except for *GGCX* 12970 C>G polymorphism who were separated on 2% agarose gel. The restriction enzymes used for each SNP and the size of digested products are summarized in Table 1.

2.6. *Statistical analysis*

Statistical analysis was performed using SPSS software (Version 18) under windows. Hardy Weinberg equilibrium was determined using a web-based calculator available at (*https://www.snpstats.net/snpstats/start.htm*) (*30*). The effect of non-genetic and genetic variants on the mean acenocoumarol dose was calculated by univariate analysis using appropriate test depending on the nature of the variables compared. Kruskal-Wallis or Mann-Whitney test was used for non-parametric analysis where appropriate. For all statistical tests, the significance level was set at 0.05. A multiple linear regression analysis was used to assess the ability of age, *VKORC1* (1173) and (1639) genotypes and *CYP2C9* haplotype to predict the dose of acenocoumarol in order to propose to clinicians an algorithm taking into account all these variables.

3. Results

3.1. *Patients characteristics*

Two hundred and seventeen patients, receiving stable dose of acenocoumarol treatment, were included in this study. The average age of the study populations was 57 ± 14 years (17-87 years old), the sex-ratio was 0.95 (111 females and 106 males), and the mean of acenocoumarol maintenance dose was 22.94 ± 12.01 mg/week. The characteristics of the included patients are summarized in the Table 2. Genotypic frequencies of all genes assessed in this study are given in Table 3. Allelic frequencies for the all assessed SNPs were found to be in Hardy-Weinberg equilibrium ($p > 0.05$) (data not shown).

Table 1. Primer design, restriction enzyme used and DNA fragments found for *VKORC1*, *CYP2C9*, *CYP4F2* and *GGCX* variations

Genetic polymorphism	Primer sequences	PCR product size	Restriction enzyme (RE)	RE digestion product size
VKORC1 (1639 G>A)	F5'GAGCCAGCAGGAGAGGGAAATAT 3' R-5'GTTTGGACTACAGGTGCCTGCC 3'	291 bp	Msp I	WT-167 + 124 bp Htz-291 + 167 + 124 bp Mut-291 bp
VKORC1 (1173 C>T)	F-5'CTAAGATGAAAAGCAGGGCCTAC3' R-5'CTGCCCGAGAAAGGTGATTTCC3'	201 bp	Sty I	WT-127 + 74 bp Htz-201 + 127 + 74 bp Mut-201 bp
*CYP2C9*2 (430 C>T)*	F-5'TCCTAGTTTCGTTTCTCTTCCTGT3' R-5'ATAGTAGTCCAGTAAGGTCAGTGA3	221 bp	Ava II	WT-122 + 99 bp Htz-221 + 122 + 99 bp Mut-221 bp
*CYP2C9*3 (1075 A>C)*	F-5'CACGAGGTCCAGAGATGCATTG3' R-5'CTTCGAAAACATGGAGTTGCAGT3'	135 bp	Nsi I	WT-116 + 19 bp Htz-135 + 116 + 19 bp Mut-135 bp
CYP4F2 (1347 G>A)	F-5′TGAAGGAGGCCTTCTCCTGACTG3′ R-5′CCAGCCTTGGAGAGACAGACAG3′	232 pb	PvuII	WT-146pb + 86 bp Htz-232 + 146pb + 86 bp Mut- 232 bp
GGCX (12970 C>G)	F-5'GCTTCTTGTTGCGAAAGCTCTAT3' R-5'CAAACACTTGGGAACAGTTAGCT3'	1288bp	Hind III	WT-1206+82 Htz-1288+ 1206pb + 82 bp Mut- 1288 bp

WT, wild type; Htz, heterozygous; Mut, homozygous mutant; bp, base pairs.

3.2. *Association of non-genetic factors with acenocoumarol dose*

The associations between age, sex, body mass index (BMI), ethnic origin, acenocoumarol therapy indication, duration of therapy, medications, the good diet monitoring and Acenocoumarol weekly dose were studied (Table 2). Univariate analysis showed that the age was the only one non-genetic factor significantly associated with the acenocoumarol weekly maintenance dose (p = 0.023) (Table 2). Patients up to 65 years (17.41 ± 10.80 mg/week) of age required a lower dose compared to the patients under 65 years old (25.25 ± 11.76 mg/week) (data not showed).

Table 2. Baseline characteristics of study population of the patients (*n* = 217) receiving Acenocoumarol therapy and the relationship of these characteristics to the maintenance weekly dose in univariate analysis

Patient details	*n* (%) or mean ± SD [min-max]*	*P* value
Age (Years)	57 ± 14 [17-87]	
< 65 Years	153 (70.5)	0.023
> 65 Years	64 (29.5)	0.000
Sex		0.056
Male	106 (48.84)	
Female	111 (51.15)	
Weight (kg)	71.85 ± 11.85	0.747
Height (cm)	169.12 ± 17.80	0.871
Body mass index (Kg/m^2)	24.52 ± 4.15 [13,95-46,77]	0.531
Ethnic		0.973
Arabic	147 (67.7)	
Berber	64 (29.5)	
Sahara	6 (2.8)	
Indication for Acenocoumarol therapy		0.321
TE disease	62 (28.58)	
AF	30 (13.82)	
Heart valves	125 (57.60)	
Duration of the therapy (Years)	6.99 ± 6.07 [1 –35]	0.56
Medication	130 (59.90)	0.131
Good diet monitoring	175 (80.64)	0.212

*Values given are mean ± standard deviation or number of participants (*n*) (%).

Table 3. Genotypic and allelic distribution of *CYP2C9*, *VKORC1*, *CYP4F2* and *GGCX* SNPs and relationship with weekly dose (mg) of Acenocoumarol

Gene and polymorphism	*n* (%)	Allele frequency	Mean dose (mg/Week) ± SD, Difference (95% CI)		*p*-value*
VKORC1-1639 G>A					
GG	57 (26.26)	G 0.54	32.00 ± 14.42	Ref.	0.0001
GA	120 (55.29)	A 0.46	21.40 ± 8.61	-10.60 (-13.90 – -7.30)	
AA	40 (18.43)		14.65 ± 8.75	-17.35 (-21.58 – -13.12)	
VKORC1-1173 C>T					
CC	64 (29)	C 0.55	32.66 ± 13.52	Ref	0.0001
CT	111 (51)	T 0.45	19.66 ± 7.93	-13.00 (-16.14 – -9.85)	
TT	42 (19)		16.81 ± 9.74	-15.85 (-19.82 – -11.87)	
*CYP2C9 *2*					
CC	161 (74.19)	C 0.87	24.56 ± 12.52	Ref	0.002
CT	55 (25.34)	T 0.13	18.49 ± 8.96	-6.07 (-9.66 – -2.48)	
TT	1 (0.01)		7	-17.56 (-40.61 – 5.49)	
*CYP2C9 *3*					
AA	191 (88.01)	A 0.94	23.48 ± 12.09		0.075
AC	26 (11.99)	C 0.06	19.00 ± 10.89	-4.48 (-9.37 – 0.42)	
*CYP2C9 Haplotypes***					
*1/*1	144 (66.35)	*1 0.81	25.05 ± 12.51	Ref	0.001
*1/*x	65 (29.95)	*x 0.19	19.05 ± 9.96	-6.00 (-9.43 – -2.58)	
*x/*x	8 (0.036)		16.63 ± 8.42	-8.42 (-16.74 – -0.10)	
CYP4F2					
GG	82 (37.78)	G 0.63	22.72 ± 12.58	Ref	0.701
GA	108 (49.76)	A 0.37	23.19 ± 11.25	0.47 (-3.00 – 3.93)	
AA	27 (12.44)		22.63 ± 13.60	-0.09 (-5.34 – 5.16)	
GGCX					
CC	205 (94.47)	C 0.97	23.05 ± 12.02	Ref	0.379
CG	12 (5.53)	G 0.03	21.00 ± 12.34	-2.05 (-9.06 – 4.95)	

Values given are number of patients (*n*) (%). *Bold indicates statistical significance. ***CYP2C9* haplotypes: *1/*1, wild-type homozygotes; *1/*x: *CYP2C9*2* or *CYP2C9*3* heterozygotes (*1/*2 and *1/*3); and *x/*x: *CYP2C9*2* homozygotes (*2/*2) or multiple heterozygotes (*2/*3).

3.3. *Association of VKROC1, CYP2C9, CYP4F2 and GGCX SNPs with the dose of acenocoumarol*

Two SNPs were investigated in the *VKORC1* gene (Table 1). Both *VKORC1* –1639 and *VKORC1* 1173 polymorphisms were observed to be affecting the weekly acenocoumarol dose significantly (p = 0.0001) (Table 3). Carriers of the GG genotype from *VKORC1* –1639 SNP requiring the highest dose (32 ± 14.42 mg/week) compared to GA carriers (21.4 ± 8.61 mg/week) and AA carriers (14.65 ± 8.75 mg/week) (Table 3). Also carriers of the CC genotype of *VKORC1* 1173 SNP requiring the highest dose (32.66 ± 13.52 mg/week) compared to CT carriers (19.66 ± 7.63 mg/week) and TT carriers (16.81 ± 9.74 mg/day) (Table 3).

Five variants (allelic combinations at two loci) were detected in Moroccan participants included in this work. A significant association was observed between the weekly maintenance acenocoumarol dose and the *CYP2C9* variants (p = 0.001) (Table 3). Patients carrying wild-type *CYP2C9* allele were found to require higher dose (25.05 ± 12.51 mg/week) than those with *CYP2C9* variant allele (19.05 ± 9.96 mg/week for the *CYP2C9* heterozygotes (*1/*2, *1/*3, *2/*3) and 16.63 ± 8.42 mg/week in mutant homozygotes patients (*2/*2) (Table 3).

There was no association found between the weekly acenocoumarol dose and the *CYP4F2* or *GGCX* variants (p-value > 0.05) (Table 3).

3.4. *Cumulate effect of VKORC1 and CYP2C9 polymorphisms on acenocoumarol dose*

The relationship between the three polymorphisms of each two SNPs of *VKORC1* gene (–1639 and 1173) and *VKORC1* –1639 or *VKORC1* 1173 variants with

Table 4. Effect of genotype combination (VKORC1 1639 and VKORC1 1173) on mean weekly dose of acenocoumarol

VKORC1 1639 G>A	*VKORC1 1173 C>T*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
GG	CC	50	34.12 ± 11.49		0.001
	CT	7	16.86 ± 9.28	-17.26 (-25.14 – -9.38)	
GA	CC	13	26.85 ± 3.11	-7.27 (-13.35 – -1.20)	0.111
	CT	91	20.55 ± 6.71	-13.57 (-17.01 – -10.13)	
	TT	16	21.81 ± 7.64	-12.31 (-17.92 – -6.70)	
AA	CC	1	35	-0.88 (-18.84 – 20.60)	0.282
	CT	13	14.92 ± 8.74	-19.20 (-25.28 – -13.12)	
	TT	26	13.73 ± 5.24	-20.39 (-25.11 – -15.67)	

Table 5. Effect of genotype combination (*VKORC1* 1639 and *CYP2C9* haplotypes) on mean weekly dose of acenocoumarol

VKORC1 1639 G>A	*CYP2C9 Haplotype*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
GG	*1/*1	42	34.76 ± 14.63		0.047
	*1/*X	13	23.69 ± 11.61	-11.07 (-17.40 – -4.73)	
	*X/*X	2	28	-6.76 (-21.21– 7.68)	
GA	*1/*1	76	22.66 ± 6.94		0.029
	*1/*X	40	19.60 ± 6.53	-3.06 (-6.96 – 0.84)	
	*X/*X	4	15.50 ± 6.50	-7.16 (-17.40 – 3.08)	
AA	*1/*1	26	16.35 ± 6.14	-18.42 (-23.40 – 13.44)	0.027
	*1/*X	12	12.17 ± 5.91	-4.18 (-11.15 – 2.79))	
	*X/*X	2	7.5 ±3 .50	-8.85 (-23.49 – 5.80)	

Table 6. Effect of genotype combination (*VKORC1* 1173 and *CYP2C9* haplotypes) on mean weekly dose of acenocoumarol

VKORC1 1173 G>A	*CYP2C9 Haplotype*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
CC	*1/*1	48	41 ± 12.81		0.011
	*1/*X	13	23 ± 7.77	-11.19 (-17.24 – -5.14)	
	*X/*X	3	11	-12.17 (-23.68 – -0.65)	
CT	*1/*1	76	24 ± 7.88		0.155
	*1/*X	40	21 ± 7.83	-1.38 (-5.25 – 2.48)	
	*X/*X	7	20 ± 5.19	-5.61 (-15.57 – 4.35)	
TT	*1/*1	29	18.59 ± 6.14		0.028
	*1/*X	12	13.58 ± 5.91	-5.00 (-11.64 – 1.64)	
	*X/*X	1	4	-14.59 (-34.27 – 5.09)	

CYP2C9 haplotypes and weekly acenocoumarol dose were analyzed, significant difference observed between the different combinations using non-parametric analysis was given in Table 4, 5 and 6. Carriers of the *VKORC1* –1639 GG and *VKORC1* 1173 CC required the highest weekly dose among all genotype combinations (42 ± 11.49 mg/week), whereas carriers *VKORC1* 1173 TT and *CYP2C9* mutant homozygotes or compound heterozygotes (*CYP2C9* *x/*x) required the lowest daily dose (4 mg/week).

3.5. *Multiple linear regression analysis*

The mean weekly acenocoumarol dose was transformed into a logarithmic dose and used as a dependent variable in univariate and multivariate analysis with age, *VKORC1* –1639, *VKORC1* 1173 genotypes and *CYP2C9* haplotype.

The results of regression analyses are summarized in Table 7. Mutation in *VKORC1* –1639, *VKORC1* 1173 SNPs, or in *CYP2C9* haplotype reduces the weekly mean dose of acenocoumarol at 25.4%, 23.4%, and 6.2% respectively. Considering all these genetic factors the R^2 for multiple regression analysis model was found to be 33.7%. Whereas the R^2 for multiple regression analysis final model was 35.9% when age variant had combined to the regression analysis with genetic factors (Table 7).

From the data in Table 7, the proposed regression equation to predict weekly dose (WD) of acenocoumarol for our final Model was:

Log (WD) = 1.925 – 0.108x (*VKORC1* 1639 G>A) – 0.073x (*VKORC1* 1173 C>T) –0.093x (*CYP2C9* Haplotype) – 0.003x (Age)

4. Discussion

The aim of this study was to investigate the impact of non-genetics factors and *VKORC1*, *CYP2C9*, *CYP4F2*, and *GGCX* polymorphisms on acenocoumarol maintenance dose in a cohort of Moroccan patients. Acenocoumarol is the only one coumarin anticoagulants available for the treatment and prevention of thromboembolic diseases in Morocco. Several factors have been described to influence the individual sensitivity for anticoagulant therapy. Genetic factors, mostly SNP variations in *CYP2C9* and *VKORC1*, in association with non-genetic factors have been reported to account for 60% of the variation in vitamin K antagonists dosage (*6*).

In our analysis, the age was the only one non-genetic factor significantly associated with the acenocoumarol dose and the required dose was significantly lower when age was increased. However, many studies have reported that the acenocoumarol and warfarin dose was influenced by other demographic factors such as gender, body mass index and ethnicity (*6,23*).

Our study showed a significant association between genetic polymorphisms in *VKORC1* and *CYP2C9* and acenocoumarol dose requirement, and corroborating with the findings reported in other countries including Morocco (*6-14,17,21,23,28,29*).

Several studies published in the world show that *VKORC*1 –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9**2

Table 7. Multiple regression models considering log-transformation the weekly dose of AC as dependent variable

Model	Composition	Unstandardized coefficients		Standardized coefficients	*p* value	95% Confidence Interval for B		Model summar		
		B	SE	Beta		Lower Bound	Upper Bound	R	R^2	Adjusted R^2
1	Constant	1.641	0.042		-	1.558	1.723			
	VKORC1 -1639	-0.176	0.021	-0.504	0.000	-0.217	-0.136	0.504	0.254	0.250
2	Constant	1.610	0.041		-	1.530	1.690			
	VKORC1 1173	-0.163	0.020	-0.483	0.000	-0.202	-0.123	0.483	0.234	0.230
3	Constant	1.464	0.046		-	1.374	1.554			
	CYP2C9 Haplotype	-0.128	0.034	-0.249	0.000	-0.196	-0.061	0.249	0.062	0.057
4	Constant	1.802	0.051		-	1.701	1.902			
	VKORC1 -1639	-0.106	0.029	-0.303	0.000	-0.163	-0.049	0.580	0.337	0.327
	VKORC1 1173	-0.085	0.028	-0.252	0.003	-0.139	-0.030			
	CYP2C9 Haplotype	-0.099	0.023	-0.235	0.000	-0.145	-0.052	0.599	0.359	0 .347
Final	Constant	1.925	0.068		-	1.791	2.059			
model	*VKORC1 1639*	-0.108	0.028	-0.309	0.000	-0.165	-0.052			
	VKORC1 1173	-0.073	0.028	-0.218	0.090	-0.128	-0.019			
	CYP2C9	-0.093	0.023	-0.221	0.000	-0.138	-0.047			
	Age	-0.003	0.001	-0.152	0.007	-0.004	-0.255			

Stepwise multivariate linear regression algorithm for prediction of stable acenocoumarol weekly dose:
Model 1: Log (WD) =1.641 – 0.176x (*VKORC1* 1639 G>A)
Model 2: Log (WD) =1.610 – 0.163x (*VKORC1* 1173 C>T)
Model 3: Log (WD) =1.464 – 0.128x (*CYP2C9* haplotype)
Model 4: Log (WD) =1.802 – 0.106x (*VKORC1* 1639 G>A) – 0.085x (*VKORC1* 1173 C>T) – 0.099x (*CYP2C9* haplotype)
Final Model: Log (WD) =1.925 – 0.108x (*VKORC1* 1639 G>A) – 0.073x (*VKORC1* 1173 C>T) – 0.093x (*CYP2C9* haplotype) – 0.003x (Age)
- Value 1 for GG; 2 for GA and 3 for AA of *VKORC1* (–1639 G>A)
- Value 1 for CC; 2 for CT and 3 for TT of *VKORC1* (1173C>T)
- Value 1 for *1/*1; 2 for *1/*2 or *1/*3 and 3 for *2/*2 or *2/*3 of *CYP2C9* haplotype
- Age in years

and *CYP2C9*3* were the most significant SNPs to affect vitamin K antagonists dosage (*6-14,17,21,23,28,29*). Our results corroborate with these facts; patients presented homozygous form of wild type allele for *VKORC1* –1639 G>A (genotype GG), *VKORC1* 1173 C>T (genotype CC), or *CYP2C9* haplotype (genotype *1/*1) required a higher acenocoumarol dose than patients in the heterozygous or homozygous variant genotypes groups. Patients with one *VKORC1* –1639 A allele need a 33.12% less dose and patients with two *VKORC*1 –1639 A alleles need a 54.21% less dose compared to patients without allele variant. This effect was also found in other populations (*23*). Similar percentages were found in Caucasian patients (25% and 50% less doses, respectively), while in Asian patients the percentages were lower (14% and 38%, respectively).

According to the previous studies investigated the effect of *VKORC1* –1639 polymorphisms on *VKORC1* expression, homozygotes for the G allele have a higher transcription activity of 44 % than those homozygote for the A allele (*31*). Therefore, homozygotes for the A allele would have lower levels of *VKORC1* expression and logically would require a lower concentration of acenocoumarol, as is the case in our study.

To our knowledge, this is the first study that reports the effect of genetic polymorphisms of the *VKORC1* 1173 C>T SNP in the variability to the acenocoumarol response among Moroccan population.

VKORC1 1173 CC genotype was associated with a significantly higher acenocoumarol dose when compared to both the CT heterozygous genotype and the TT variant genotype. Patients carrying CT or TT genotypes required a 39.80% and 48.53% less dose, respectively, compared to patients with a wild genotype (CC genotype). These results are demonstrated in other countries using acenocoumarol, in the German and Austrian populations (25% and 52%) (*32*), in Dutch patients (28% and 47%) (*24*), and in Chinese patients (33% and 55%) (*33*).

Like Warfarin, acenocoumarol is metabolized by *CYP2C9*. It has been identified that *CYP2C9*2* and *CYP2C9*3* variants alleles reduce in vitro enzymatic activity. Therefore, patients carrying heterozygous and homozygous variants *CYP2C9* alleles were more sensitive to vitamin K antagonists therapy and present a higher risk of bleeding complications than patients carrying the *CYP2C9*1* alleles (*34*). Here, patients with one *CYP2C9* variant allele (*1/*2 or *1/*3) required a 23.95% less dose and patients with two variant alleles (*2/*2, *2/*3) a 33.61% less dose than patients without this variant allele (*1/*1). These results correlate with previous findings indicating that acenocoumarol dose requirement is 19-29% less in carriers one or two *CYP2C9* variant allele than in wild-types (*35*).

The data reported by Smires *et al*. has shown that, there was no association between the acenocoumarol dose variation and the *CYP4F2* polymorphisms among Moroccan population (*28*). Although, the *CYP4F2* variant allele (A) frequency was of 37% (Table 3), our findings support this fact and confirm that the required acenocoumarol dose, is probably not affected by the modification of the *CYP4F2* gene in the Moroccan population.

This study is the first in Morocco to assess whether the genetic polymorphisms of *GGCX* SNP influences the individual response for AC therapy. However, our results shown that there was no association between *GGCX* polymorphisms and acenocoumarol dose maintenance.

The *GGCX* wild type allele (C) was found in a very higher frequency (97%) in our study samples (Table 3). It's known that ethnic differences in allelic frequencies of SNPs implicated in vitamin K antagonists dose variability affect the sensitivity to treatment among different population (*36*). Thus, we can't confirm that the lower frequency of *GGCX* variant allele (G) was responsible for such a result.

However, a large and representative investigation is necessary to better evaluate the effect of *CYP4F2* and *GGCX* SNPs on the acenocoumarol dose assessment among Moroccan population.

Similar to the results from other studies (*12,37*), non-parametric analysis based on acenocoumarol dose, *VKORC1* –1639, *VKORC1* 1173 SNPs and *CYP2C9* haplotypes demonstrated that these three SNPs exert a cumulative effect on the acenocoumarol dose requirement. Patients carrying homozygous variant alleles at *VKORC1* –1639 (AA) and *VKORC1* 1173 (TT) SNPs required 59.75% less dose than wild type for the two SNPs. Carriers *VKORC1* –1639 AA and *CYP2C9* *x/*x genotypes required 78.42% less dose than *VKORC1* –1639 GG and *CYP2C9 *1/*1* genotypes. The single patient who had *VKORC1* –1173 TT variant and *CYP2C9* *x/*x haplotype (*CYP2C9* *2/*2 or *2/*3) had the lowest weekly AC dose (4 mg/week) among the all investigated patients (90.24% less dose).

As reported in other investigations (*33,38-40*), the multiple linear regression analysis confirmed that *VKORC1* and *CYP2C9* contribute mainly to the interindividual variability of the acenocoumarol dose requirement. Our multivariate model including *VKORC1* –1639, *VKORC1* 1173 genotypes and *CYP2C9* haplotype accounted for 33.7% (R^2 = 0.337) of our total observed variation. While our multivariate final model including age, *VKORC1* –1639, *VKORC1* 1173 and *CYP2C9* was 35.9%. The same R^2 value is approximately reported by Dhakchinamoorthi *et al*. (30.4%) (*41*) and Rathore *et al*. (41%) (*42*). However, high values were explained in the algorithms developed by Borobia *et al*. (61%) (*39*) and Markatos *et al*. (55%) (*43*) (Table 8). We can speculate that the R^2 of the multivariate regression model is affected by the nature and number of independent variables.

As shown by the results of this and other studies (*6-14,17,21,23,28,29*), the variation in acenocoumarol dose response is partially influenced by the differences

Table 8. Published algorithms to predict the required acenocoumarol dose

Reference	Country	Genetic parameter	Clinical parameter	R^2 %
Cerezo-Manchado J *et al*. 2013 (*40*)	Spain	*CYP2C9, VKORC1, CYP4F2*	Age, BSA, gender	50
Dhakchinamoorthi K *et al*. 2013 (*41*)	India	*CYP2C9 VKORC1*	Age BMI	30.4
Rathore SS *et al*. 2012 (*42*)	India	*CYP2C9, VKORC1, CYP4F2, GGCX*	Age, weight, height, BSA, gender	41
Borobia A *et al*. 2012 (*39*)	Spain	*CYP2C9, VKORC1, CYP4F2, APOE*	Age, BMI, CM	61
Markatos C *et al*. 2008 (*43*)	Greece	*CYP2C9 VKORC1*	Age, gender, CM	55
Van Schie R *et al*. 2011 (*38*)	Netherlands	*CYP2C9 VKORC1*	Age, height, weight, gender, CM	53
Present Study 2017	Morocco	*CYP2C9 VKORC1*	Age	35.9

in genetic polymorphisms, especially in *CYP2C9* and *VKORC1*. Therefore, our proposed regression equation might be useful to Moroccan clinician physicians for initial determination of acenocoumarol dose. The effectiveness of this equation should be evaluated in a future study.

In conclusion, the present study confirmed that the high variability in the maintenance dose of acenocoumarol in Moroccan populations is related to demographic factors and genetic factors, in particular *VKORC1* –1639, *VKORC1* 1173, *CYP2C9*2* and *CYP2C9*3*. In addition, acenocoumarol susceptibility was shown to be higher in patients with one or more mutations in these SNP. The presence of these mutations indicates that a lower initial dose of acenocoumarol should be used to reduce the risk of bleeding. Furthermore, this study provides a useful model for predicting the weekly dose of acenocoumarol for Moroccan patients based on their genetic makeup.

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