Towards improvement of oral anticoagulant therapy

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CHAPTER 4

Effects of CYP2C9 and VKORC1 on INR variations and dose requirements during the initial phase of anticoagulant therapy with acenocoumarol

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Abstract

***Introduction*:** Anticoagulants of the coumarin type are effective drugs for the treatment and prevention of thromboembolic diseases. However, they have a narrow therapeutic range and show interindividual and intraindividual variability in dose requirement, largely conditioned by both environmental and genetic factors.

Methods: This prospective study investigated, during the initial phase of acenocoumarol therapy, the effect of *CYP2C9* variant alleles and *VKORC1* haplotypes, single and in combination, in 220 Italians.

Results: CYP2C9*3 was associated with a 25% dose reduction and an increased risk of over-anticoagulation (INR>6) on day 4. Two copies of the VKORC1*2 haplotype were associated with a 45% dose reduction and an increased risk of over-anticoagulation. Homozygosity for VKORC1*3 and VKORC1*4 was associated with an increased dose requirement and a reduced risk of over-anticoagulation. The VKORC1*3 or *4 plus CYP2C9*1 genotype combination was associated with the highest dose requirement and the lowest INR on day 4; VKORC1*2 plus CYP2C9*3 was associated with the lowest dose requirement, the highest INR and an increased risk of over-anticoagulation. Even though they spent approximately 50% of the time within the target therapeutic range, VKORC1*3 or *4 plus CYP2C9*1 carriers spent a large percentage of the remaining time below and carriers of VKORC1*2 plus CYP2C9*3 above the target range.

Discussion: The determination of VKORC1*3 and VKORC1*4 haplotypes may be an important addition to *CYP2C9* and VKORC1*2 genotyping to identify patients at risk of being outside the target range during initial anticoagulation with acenocoumarol.

Introduction

Anticoagulant drugs play an important role in the prophylaxis and treatment of thrombotic events, and are widely used chronically or intermittently in cardiovascular medicine and surgery [1]. For instance, their use has decreased the risk of thrombotic events and death due to acute myocardial infarction by 24% [2] and 80%, respectively [3]. However, the use of vitamin K (VitK) antagonists as oral anticoagulants still poses significant clinical challenges because their therapeutic index is narrow and dose-response relationship unpredictable [4]. The latter situation makes difficult to predict the daily maintenance dose, that for warfarin may range from as little as 0.5 to as much as 60 mg [5] and for acenocoumarol from 1 to 56 mg [6]. These large variations in dose requirements, influenced by pharmacokinetic and pharmacodynamic aspects in turn determined by genetic and environmental factors, demand frequent measurements of the International Normalized Ratio (INR) of the prothrombin time to evaluate the degree of anticoagulation and to assess the need for dosage changing.

Warfarin is the main vit.K antagonist prescribed in the United Kingdom and North-America, while acenocoumarol, the 4'nitro-analogue of warfarin, is widely used in European countries. Elimination of the two acenocoumarol isomers depends entirely on their hepatic biotransformation by the CYP2C9 enzyme [7, 8]. Carriers of genetic polymorphisms of *CYP2C9*, in particular of the CYP2C9*2 and CYP2C9*3 variant alleles [9-10], require smaller doses of acenocoumarol than carriers of the most frequent 2C9*1 wild-type allele and have a higher incidence of minor bleeding episodes [11], even though dose-effect association for the CYP2C9*2 variant allele was not consistently found [6, 11-17]. The CYP2C9*3 variant allele is also associated with a reduced likelihood to achive stability within the target INR range during the first 6 months of acenocoumarol use and with an increased risk of severe anticoagulation (INR>6) [14].

Oral anticoagulants exert their effect by reducing the regeneration of Vit.K from its epoxide through the inhibition of Vit.K epoxide reductase [18]. This protein is coded by the Vit.K epoxide reductase complex subunit 1 gene (*VKORC1*) [19-20]. Several polymorphisms in the *VKORC1* gene are mainly located in non-coding regions and are in strong linkage disequilibrium (LD), so that a few of them are enough to infer the most common haplotypes that explain the genetic variability of *VKORC1* [21, 22]. Rieder *et al.,* [21] divided the most common haplotypes in two main groups: group A (haplotypes H1 and H2), associated with low warfarin dose requirement and the group B (haplotypes H7-H9), associated with an increased dose requirement. Geisen *et al.*, [22] later described three haplotypes (VKORC1*2, VKORC1*3, VKORC1*4) covering >99% of the genetic variability in Europeans: the VKORC1*2 haplotype (corresponding to group A) was strongly associated with an increased coumarin sensititity whereas VKORC1*3 and VKORC1*4 (both included into group B) with partial resistance. Even though the combined effect of both *VKORC1* and *CYP2C9* on warfarin [21, 23- 27] was fully investigated, less information is available on acenocoumarol response [15, 28-30]. Thus, this prospective study was designed to investigate the contribution of the common *CYP2C9* variant alleles and *VKORC1* haplotypes in the modulation of acenocoumarol response. We chose to investigate the initial phase of treatment because the first months are particularly problematic, since the safe and effective dose for an individual patient is not known and is determined empirically, with an increased risk of over-anticoagulation and hemorrhagic complications [31]. Our primary purpose was to evaluate the effect of the *CYP2C9* variant alleles and *VKORC1* haplotypes and of their combination on the first INR determination and on mean weekly acenocoumarol dosages. The risk of severe over-anticoagulation (defined as INR>6) at the time of the first INR

determination and the percentage of time spent below, within and above the target INR were also analysed.

Methods

Patients

The study cohort of North-Italian patients was prospectively recruited at two anticoagulant clinics in Milan: the Istituto Clinico Humanitas and San Paolo Hospital. Included were patients aged 18 years or more who started oral anticoagulant therapy with acenocoumarol for arterial or venous thrombosis or non-ischemic heart disease (atrial fibrillation and dilatative cardiopathy). Exclusion criteria were the presence of concomitant severe diseases known to interfere with this treatment (i.e. liver cirrhosis, uremia, malignant diseases). All patients started treatment with the same standard dosage of acenocoumarol during the first 3 days of therapy (4 mg, 4 mg, 2 mg), the first INR being scheduled on day 4 and subsequently once or twice weekly. Acenocoumarol dosing was based on a computerized system (Parma 4.1) which includes an algorithm that recommends the therapeutic dose [32]. Demographic data, indication for anticoagulant therapy, INR target range, concomitant disease, co-medications, diet (vegetarian or not) and bleeding episodes were collected. The daily acenocoumarol doses administered during the initial phase of treatment (arbitrarily set at 52 days, i.e., first 3 days plus 7 weeks) and INR values obtained during control visits at the clinic were also collected. All patients gave written informed consent for analysis of their DNA. The study protocol was approved by the Medical ethics Committee of the participating hospitals

Laboratory methods

Venous blood was collected in sodium citrate 0.105 M. Plasma and cells were separated after centrifugation at 4000 g for 30 min. DNA was isolated from leukocytes using the salting out method [33]. The INRs (International Normalized Ratio) were performed in all centers using the same reagent/instrument combination, namely, human recombinant thromboplastin (RecombiPlasTin, HemosILTM, INstrumentation Laboratory Company, USA), and a Electra 1600 coagulometer (IL, Milan, Italy). All the participating centers performed regular external quality control exercises.

Genotyping

The polymerase chain reactions and endonuclease digestions were used for the detection of the *CYP2C9*2* (c.430 C/T, rs1799853) and *2C9*3* (c.1075 A/T, rs1057910) variant alleles [34]. Three polymorphisms located at the VKORC1 gene, i.e. c.173+1000 C/T (rs9934438) in intron 1 and c.492+134 G/A (rs7294) in the 3'UTR, both previously identified by D'Andrea *et al.,* [35] and c.173+525 C/T (rs17708472) were analysed. For the analysis of VKORC1 c.173+1000 C/T a sense (5'-TGACATGGAATCCTGACGTG -3') and antisense (5'-GAGCTGACCAA-GGGGGAT-3') PCR primers and *Hinf*I restriction enzyme (New England BioLabs) were used; for the c.492+134 G/A a sense (5'-ATGGAGTGTTCGGG-AGGTG-3') and antisense (5'-ACAGTCCATGGCAGACACAT-3') PCR primers and *Aci*I restriction enzyme (New England BioLabs), and for that of the c.173+525 C/T a sense (5'-CGTTAGCATAATGACGGAATACAG-3') and antisense (5'-AACTCCTGACTTCAAGTGATCCAT-3') PCR primers and *Bfa*I restriction enzyme (New England BioLabs) were used, respectively.

The analysis of the combination of these polymorphisms allow to establish for each patient the presence of the VKORC1*1 haplotype, (the putative ancestral

haplotype, characterized by the presence of the normal alleles: c.173+1000 C, c.492+134 G and c.173+525 C polymorphisms [22]), the VKORC1*2 haplotype (characterized by the presence of the variant allele c.173+1000 T and the normal alleles: c.492+134 G and c.173+525 C [22]), the VKORC1*3 haplotype (characterized by the presence of the variant allele c.492+134 A and the normal alleles: c.173+1000 C and c.173+525 C [22]), and the VKORC1*4 haplotype (characterized by the presence of the variant allele c.173+525 T and the normal alleles: c.173+1000 C and c.492+134 G [22]).

Statistical analysis

Continuous variables, expressed as mean (range) were used for age, body surface area (BSA), days of follow-up, INR range, INR values and administered daily doses. BSA was calculated according to the formula: $0.00718 \times \text{height}^{0.725} \times \text{weight}^{0.425}$ [36]. In each patient the doses of acenocoumarol, administered after the first 3 days of standard dosages (4 mg, 4mg, 2 mg), were calculated for weekly periods (day 4-10, day 11-17, day 18-24, day 25-31, day 32-38, day 39-45, day 45-53). In order to analyze the effect of age on dose requirement, age at the start of therapy was divided into seven 10-year categories, the lowest ranging from years 20 to 30 and the highest from years 80 to 90. Dummy variables were used to code for sex, clinical variables (indications for acenocoumarol, presence of concomitant diseases) and concomitant medications. In order to quantify how a single copy or two copies of each *CYP2C9* or *VKORC1* variant alleles affect acenocoumarol dose requirement (according to a additive allelic effect), dummy variable codes were established. As regards *CYP2C9*, 0 was assigned to carriers of two copies of the CYP2C9*1 wild-type allele, 1 to carriers of one copy of the CYP2C9*2 variant allele, 2 to carriers of two copies of the CYP2C9*2 variant allele, 3 to carriers of one copy of the CYP2C9*3 variant allele, 4 to carriers of both CYP2C9*2 and

CYP2C9*3 variant alleles and 5 to carriers of two copies of the CYP2C9*3 variant allele. Patients were then divided into groups according to the presence of two wild-type alleles (code: 0), at least one CYP2C9*2 variant allele (heterozygous or homozygous, code: 1) or at least one CYP2C9*3 variant allele (heterozygous or homozygous, code: 2). Heterozygotes for the 2C9*2/*3 genotype were grouped only with patients carrying at least one 2C9*3 variant allele.

As regards *VKORC1*, 0 was assigned to carriers of two copies of the VKORC1*1 ancestral haplotype, 1 to carriers of one copy of the VKORC1*3 haplotype, 2 to carriers of one copy of the VKORC1*4 haplotype, 3 to carriers of two copies of the VKORC1*3 haplotype, 4 to carriers of both VKORC1*3 and VKORC1*4 haplotype, 5 to carriers of two copies of the VKORC1*4 haplotype, 6 to carriers of both VKORC1*2 and VKORC1*3 haplotype, 7 to carriers of both VKORC1*2 and VKORC1*4 haplotype, 8 to carriers of one copy of the VKORC1*2 haplotype and 9 to carriers of two copies of the VKORC1*2 haplotype. The ascending number of the dummy variable codes was assigned to each genotype according to the increased reported effect of the variant alleles on coumarin response [22]. Each group was compared with the wild-type group. Finally, patients were divided according to the possible *CYP2C9* and *VKORC1* genotype combinations.

In order to analyze the effect of *CYP2C9* variant alleles and *VKORC1* haplotypes on dose requirement during the first 7 weeks of therapy, only patients with target INR between 2.0 and 3.0 were considered, while patients with a different INR target were excluded from this analysis. To analyze the effect of *CYP2C9* variant alleles and *VKORC1* haplotypes on the first INR, only patients who had INR measured on day 4 of treatment were considered.

For comparison of the mean INR value on day 4 of therapy and the mean dose requirement during the first 7 weeks of therapy between single and combined

genotypes, one-way ANOVA tests with post-hoc multiple comparison adjustments (Bonferroni for equal variance or Dunnett T3 for non-equal variance) were performed. A multivariate linear regression model was used to assess the independent effect of genetic factors and other environmental variables on the first INR estimate and dose requirement. Relative risks (RR) with 95% confidence interval (CI) were calculated to compare the proportion of patients with INR>6 (severe over-anticoagulation) for single and combined genotypes. Finally, for each genotype combination, the percentage of time (days) spent within, above and below the INR target range during the first 7 weeks of therapy were evaluated using the linear interpolation method described by Rosendaal *et al.* [37]. One-way ANOVA tests with post-hoc multiple comparison adjustments (Bonferroni for equal variance or Dunnett T3 for non-equal variance) were performed to assess the differences in percentages between genotype combinations. 95% confidence intervals (CI) were used to indicate the significance of each test. All statistical analysis were performed using the SPSS software (version 11.5, SPSS Inc, , Chicago, III).

Results

Between 2003 and 2005, 220 Italian patients (all Caucasians) meeting the inclusion criteria were enrolled in the study. Patient characteristics are summarized in Table 1. Two hundred and one (91.4%) completed the scheduled follow-up of 53 days, whereas 19 did not complete follow up or stopped therapy for various reasons. Allele and genotype frequencies for the CYP2C9*2 and CYP2C9*3 variant alleles and for the different VKORC1 haplotypes are summarized in Table 2. None of the patients was homozygous for the reference VKORC1*1 haplotype.

Table 1. Characteristics of patients

Number of patients	220
Age, years [mean,(range)]	65 (23 – 87)
Gender, n (%)	
Male	133 (60.5)
Female	87 (39.5)
BSA, m^2 [mean, (range)]	1.84 (1.36-2.48)
Follow-up, days [median, (range)]	53 (6 - 53)
Indication for anticoagulant therapy, n (%):	
Arterial thrombosis*	39 (17.7)
Venous thrombosis**	57 (25.9)
Non-ischemic cardiac disease***	124 (56.4)
Target INR, n (%):	
1,8 – 2,2	1 (0.5)
1,8 – 2,5	1 (0.5)
2,0 – 2,5	2 (0.9)
2,0 – 3,0	187 (85.0)
2,5 – 3,5	28 (12.7)
2,5 – 5,0	1 (0.5)
Concomitant diseases, n (%):	
cardiovascular risk factors	73 (33.2)
history of arterial thrombosis	17 (7.7)
history of venous thrombosis	4 (1.8)
benign tumors	7 (3.2)
diseases associated with haemorragic risk	2 (0.9)
venous insufficiency of the lower limb	2 (0.9)
Concomitant medications, n (%):	
Drugs known to increase the INR	44 (20.0)
Drugs known to decrease the INR	17 (7.7)
Drugs with unknown effect on INR	109 (49.5)

* Arterial thrombosis includes acute coronary syndromes, stroke, TIA, peripheral arterial emboli
** Venous thrombosis includes deep vein thrombosis and pulmonary embolism
*** Non-ischemic cardiac disease includes atrial fibrillation and dilatative cardiomyopathy

Table 2. Genetic characteristics of patients

Polymorphism	Allele	%	Genotype	N	%
CYP2C9	*1	76.1	*1/*1	132	60.0
	*2	14.8	*1/*2	48	21.8
	*3	9.1	*1/*3	25	11.4
			*2/*2	6	2.7
			*3/*3	4	1.8
			*2/*3	5	2.3
VKORC1	*1	3.35	*1/*1	0	0
	*2	42.63	*1/*3	8	3.6
	*3	36.16	*1/*4	4	1.8
	*4	17.86	*3/*3 [a]	29	13.2
			*3/*4	32	14.5
			*4/*4 [d, e]	6	2.7
			*2/*3	60	27.3
			*2/*4	30	13.6
			*1/*2	3	1.4
			*2/*2 [b, c]	48	21.8

[a] 1 patient was VKORC1 *3/*3 homozygous + *1/*4 heterozygous, [b] 1 patient was VKORC1 *2/*2 homozygous + *1/*3 heterozygous, [c] 1 patient was VKORC1 *2/*2 homozygous + *1/*4 heterozygous, [d] 2 patients were VKORC1 *4/*4 homozygous + *1/*2 heterozygous, [e] 3 patients were VKORC1 *4/*4 homozygous + *1/*3 heterozygous

Effect of single CYP2C9 alleles and VKORC1 haplotypes on the first INR value

This effect was evaluated only in patients (n=164, 74.5%) who had their INR measured on day 4 of treatment. Table 3 shows that carriers of at least one CYP2C9*3 variant allele had a higher mean first INR than CYP2C9*1/*1 wild-type patients, the highest INR being observed for the homozygous CYP2C9*3/*3 genotype [5.6; range: 3.2-10.0; 95%CI of difference *vs* wild-type patients: -7.68 - 2.14]. The presence of at least one CYP2C9*2 variant allele resulted in a higher INR than that of wild-type patients too.

As the reference VKORC1*1/*1 genotype was not represented, each *VKORC1* genotype was compared to VKORC1*2 homozygotes, being the VKORC1*2 haplotype associated with the larger INR variation and the lower warfarin maintenance doses [38]. Homozygotes for the VKORC1*2 haplotype had the highest mean first INR compared to all the other genotypes (Table 3). Homozygotes for the VKORC1*3 haplotype had no difference in mean INR values on day 4 compared to homozygotes for the VKORC1*4 haplotype (2.6; range: 1.4-7.7 vs 2.4, range: 1.4-3.6; 95%CI of diff: -2.14 – 2.32). Heterozygous combinations of the VKORC1*3 or VKORC1*4 haplotypes seem to have at least equal impact on the INR value on day 4 as homozygosity for either VKORC1*3 or VKORC1*4. On the other hand, also VKORC1*1/*2 heterozygotes had higher mean first INR.

The risk of early over-anticoagulation (INR >6 on day 4) associated with the presence of variant alleles was evaluated in comparison to CYP2C9*1/*1 homozygotes and to homozygotes for the VKORC1*2 haplotype. Table 3 shows that carriers of at least one CYP2C9*2 or CYP2C9*3 variant allele had 3.39 and 2.81 times higher risks of over-anticoagulation. The highest risk (6.58 times) was found for CYP2C9*3/*3 homozygotes (95%CI: 0.93 - 45.45). The homozygous VKORC1*2/*2 genotype was characterized by the highest number of INR>6 on day 4 (Table 3).

Table 3. Relative risk (RR) and 95% confidence intervals (CI) of INR>6 on day 4 of acenocoumarol therapy in carriers of variant *CYP2C9* alleles or *VKORC1* haplotypes. References were patients with the *CYP2C9* wild-type genotype and those homozygous for the *VKORC1*2* haplotype, respectively.

		n° of INR at day 4	mean INR on day 4 (range)	p (2-tailed)	[95% CI]	n° INR>6 on day 4	RR [95% CI]
	*1/*1	105	2.9 (1.0 - 6.8)	1.0 (ref)	0 (ref)	4	1.0 (ref)
CYP2C9	*2/-	31	3.3 (1.5 - 7.8)	0.42	-0.37–1.32	4	3.39 [0.90 – 12.82]
	*3/-	28	3.7 (1.2 – 10.0)	0.079	-0.07–1.79	3	2.81 [0.67 – 11.90]
	*2/*2	32	4.5 (2.2 – 10.0)	1.0 (ref)	0 (ref)	7	1.0 (ref)
	*3/*3	21	2.6 (1.4 - 7.7)	0.002	0.47–3.42	1	0.22 [0.03 – 1.64]
	*4/*4	5	2.3 (1.4 - 3.6)	0.037	0.10–4.14	0	no risk estimate
	*1/*2	2	3.8 (3.3 - 4.3)	0.97	-7.05–8.46	0	no risk estimate
VKORC1	*1/*3	7	2.8 (1.1 - 5.9)	0.53	-1.29–4.72	0	no risk estimate
	*1/*4	4	2.2 (1.7 - 3.0)	0.008	0.53–4.14	0	no risk estimate
	*2/*3	44	2.9 (1.0 - 6.9)	0.003	0.34–2.82	1	0.10 [0.01 – 0.80]
	*2/*4	26	3.1 (1.0 - 6.7)	0.048	0.01–2.88	2	0.35 [0.08 – 1.55]
	*3/*4	23	2.4 (1.0 - 4.9)	0.000	0.82–3.38	0	no risk estimate

Table 4: Mean weekly dose (SD) and 95% CI of acenocoumarol in carriers of variant *CYP2C9* or *VKORC1*.

			Week 1 (days 4-10)	Week 2 (days 11-17)	Week 3 (days 18-24)	Week 4 (days 25-31)	Week 5 (days 32-38)	Week 6 (days 39-45)	Week 7 (days 46-53)
CYP2C9	***1/*1**	mean mg/week (SD)	14.3 (6.8)	14.6 (6.4)	15.4 (7.2)	15.7 (7.5)	15.6 (7.9)	15.9 (7.9)	19.0 (9.1)
	n=116	95% CI	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	***2/-**	mean mg/week (SD)	13.0 (5.4)	14.1 (6.5)	13.6 (7.6)	13.5 (7.1)	13.7 (7.1)	13.8 (7.4)	15.8 (8.6)
	n=45	95% CI	-1.2-3.8	-2.1-3.2	-1.3-4.8	-0.9-5.3	-1.3-5.1	-1.2-5.3	-0.8-7.0
	***3/-**	mean mg/week (SD)	11.2 (4.6)	11.0 (5.0)	11.5 (4.3)	11.1 (4.0)	11.4 (4.5)	11.5 (4.7)	14.1 (5.3)
	n=26	95% CI	0.4-5.9	0.3-6.9	0.1-7.6	0.8-8.3	0.2-8.2	0.3-8.4	0.2-9.5
VKORC1	***1/*2**	mean mg/week (SD)	10.7 (4.6)	11.0 (3.6)	17.0 (17.4)	11.0 (5.3)	10.7 (4.6)	12.0 (5.2)	14.0 (4.4)
	n=3	95% CI	-27.9-25.5	-20.2-16.7	-20.4-5.7	-30.9-27.9	-25.7-23.8	-31.1-26.1	-24.3-18.9
	***1/*3**	mean mg/week (SD)	11.5 (7.0)	13.7 (9.1)	15.5 (9.1)	15.1 (8.5)	15.8 (9.0)	16.3 (9.6)	21.5 (8.9)
	n=7	95% CI	-14.8-10.6	-20.9-12.1	-14.8-3.0	-20.9-9.7	-22.3-10.1	-24.3-10.6	-28.6-8.3
	***1/*4**	mean mg/week (SD)	14.7 (3.1)	18.0 (4.2)	19.0 (7.1)	18.5 (5.0)	16.0 (1.4)	15.5 (0.7)	18.0 (1.4)
	n=3	95% CI	-21.6-11.2	-97.6-80.1	-25.2-6.4	-117.8-99.8	-16.7-4.1	2.2-9.8	-15.3-1.9
	***2/*2**	mean mg/week (SD)	9.5 (3.3)	9.3 (4.7)	9.6 (4.9)	9.5 (4.8)	9.7 (5.0)	9.5 (4.9)	11.4 (5.9)
	N=41	95% CI	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	***3/*3**	mean mg/week (SD)	16.0 (5.9)	16.0 (5.7)	16.4 (6.9)	17.5 (7.9)	18.0 (9.0)	18.2 (8.6)	22.5 (9.7)
	n=26	95% CI	2.2-10.8	2.2-11.2	1.32-12.2	2.0-14.0	4.1-16.0	2.1-15.4	3.2-19.1
	***4/*4**	mean mg/week (SD)	15.8 (3.2)	17.3 (3.4)	15.5 (4.8)	15.0 (5.4)	16.0 (5.1)	16.2 (5.3)	18.6 (6.4)
	n=6	95% CI	-12.9-0.1	1.1-15.0	-15.4-3.7	-16.6-5.6	-16.7-4.1	-17.6-4.3	-23.2-8.7
	***2/*3**	mean mg/week (SD)	14.6 (7.5)	15.4 (6.6)	15.9 (7.4)	16.2 (7.3)	15.9 (7.4)	16.2 (7.7)	19.1 (8.7)
	n=48	95% CI	1.2-9.1	2.2-10.1	1.6-10.9	2.4-11.1	1.8-10.6	2.1-11.3	2.3-13.2
	***2/*4**	mean mg/week (SD)	14.0 (6.0)	14.4 (5.9)	14.3 (6.1)	14.0 (6.6)	14.0 (7.0)	14.4 (7.2)	16.4 (7.9)
	n=26	95% CI	0.6-8.9	0.4-9.8	-10.2-1.0	-9.8-0.8	-9.9-1.4	-10.7-0.9	-11.9-1.8
	***3/*4**	mean mg/week (SD)	15.5 (6.2)	15.8 (5.7)	16.3 (6.3)	16.8 (6.5)	17.0 (6.7)	17.1 (6.6)	19.6 (8.1)
	n=27	95% CI	1.6-10.5	2.1-11.0	1.2-12.1	2.2-12.5	1.9-12.6	2.3-13.0	1.8-14.8

Sex, indication for therapy, concomitant diseases, medications and BSA did not affect the first INR. Age at start of therapy was positively associated with the first INR, but was not a confounding factor for the association between genotype and first INR. Multiple linear regression analysis showed that both *CYP2C9* and *VKORC1* genotypes separately influenced the first INR value on day 4 of therapy and that, together with age, they explain 26% of the observed INR variability.

Effect of single CYP2C9 alleles and VKORC1 haplotypes on dose requirement during the first 7 weeks of therapy

This effect was evaluated in 187 patients (85%, only patients with a target INR=2.0-3.0). Table 4 shows that carriership of at least one CYP2C9*2 allele was associated with a lower mean weekly dose compared to patients carrying only the wild-type allele. The decrease varied from 9% in the first week (1.3 mg) to 17% in the 7th week (3.13 mg). Carriership of at least one CYP2C9*3 variant allele decreased to a greater degree than CYP2C9*2 the weekly dose compared to wild-type patients, i.e. from 22% in the first week of therapy (3.1 mg) to 25% in the 7th week (4.8 mg).

Homozygosity for the VKORC1*2 haplotype was associated with a lower mean weekly dose compared to the other *VKORC1* genotypes, particularly in comparison with carriers of the homozygous VKORC1*3 haplotype, the decrease varying from 41% in the first week (6.5 mg) to 49% in the 7th week (11.1 mg), and with carriers of the homozygous VKORC1*4 haplotype, the decrease being around 40% all along the first seven weeks (~ 7 mg). Homozygotes for the VKORC1*3 and VKORC1*4 haplotypes showed no differences in the required mean weekly doses [95% CI: -22.59 – 29.92 at week 7]. All the other *VKORC1* genotypes required mean weekly doses in the range between those required by

VKORC1*2/*2 and VKORC1*3/*3.

Concomitant medications and diseases were not associated with dose requirement, whereas BSA, age, sex and indication for therapy were associated factors but not confounders for the association between genotype and dose requirement. Multiple linear regression analysis showed that *CYP2C9* and *VKORC1* genotypes, together with sex, BSA, age at the start of therapy and indication for therapy, accounted for 20% of the observed variability in dose requirement in the first week of therapy. The influence of these factors increased over the following weeks and in the 7th week they accounted for 27% of the variability. The major role on variability was exerted by *VKORC1,* that in the 7th week of therapy accounted for 12% of it, followed by *CYP2C9* genotype (5%).

Effect of CYP2C9 and VKORC1 combinations on the first INR value

Only patients homozygous for the VKORC1*2, VKORC1*3 and VKORC1*4 haplotypes were considered. As VKORC1*3 and VKORC1*4 haplotypes showed no differences in the first INR and dose requirement, homozygous carriers of these haplotypes were combined. In each of the VKORC1*2 and VKORC1*3 or *4 groups, the effect of the CYP2C9*2 and *3 variant alleles were investigated.

Table 5. Frequency of the combinations between homozygous VKORC1*2 and VKORC1*3 or VKORC1*4 haplotypes and *CYP2C9* alleles in the whole cohort of patients.

GENOTYPE COMBINATION	N of patients from the whole cohort	%	N of patients with target INR of 2.0-3.0	%
VKORC1*2 + CYP2C9*1	31	37.4	27	37
VKORC1*2 + CYP2C9*2	11	13.3	9	12.3
VKORC1*2 + CYP2C9*3	6	7.2	5	6.8
VKORC1*3 or *4 + CYP2C9*1	19	22.9	18	24.7
VKORC1*3 or *4 + CYP2C9*2	9	10.8	7	9.6
VKORC1*3 or *4 + CYP2C9*3	7	8.4	7	9.6
TOTAL	83	100.0	73	100.0

The frequency of each genotype combination in the whole cohort of patients is shown in Table 5.

Figure 1 shows the first INR in patients with different *VKORC1* and *CYP2C9* genotype combinations. Among carriers of VKORC1*2, associated with the highest mean INR on day 4, the presence of the CYP2C9*3 variant allele, but not that of the CYP2C9*2 variant allele, increased the mean INR on day 4 compared to carriers of the CYP2C9*1 wild-type allele [5.9; range: 3.6–10.0 vs 4.1; range: 2.2-6.6; 95%CI of diff: -0.36 – 3.99]. The CYP2C9*3 variant allele was also associated with a 5 times increased risk of INR>6.0 [95%CI: 1.07–23.26] compared to the CYP2C9*1 wild-type allele.

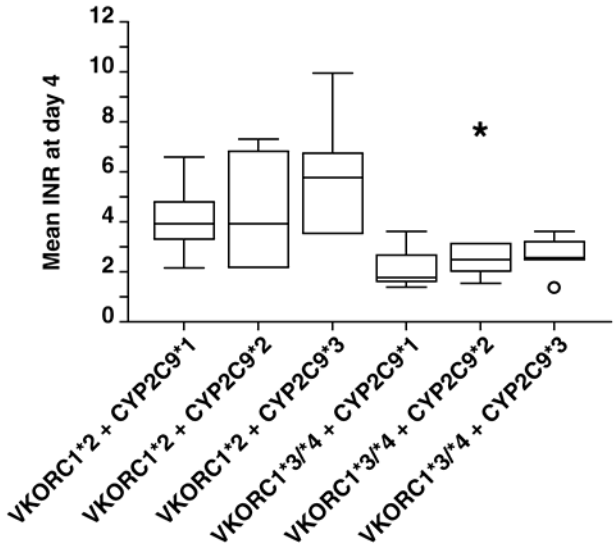

Figure 1. Box plot of first INR values on day 4 of acenocoumarol and therapy in patients with different C4P2C9 and VKORC1 genotype combinations.

Among carriers of VKORC1*3 or VKORC1*4, the presence of both the CYP2C9*2 and CYP2C9*3 variant alleles did not affect the mean INR on day 4 compared to carriers of the CYP2C9*1 wild-type allele [3.3; range: 1.5-7.8 vs 2.2;

range: 1.4-3.6; 95%CI of diff: -1.18 – 3.34 and 2.7; range: 1.4-3.6 vs 2.2; range: 1.4-3.6; 95%CI of diff: -1.92 – 2.91, respectively].

Effect of CYP2C9 and VKORC1 combinations on weekly dose requirement

Weekly dose requirement was compared only in patients with target INR of 2.0 to 3.0 (n=73). The doses of acenocoumarol required by patients with different *CYP2C9* and *VKORC1* genotype combinations in the first 7 weeks of treatment are shown in Figure 2.

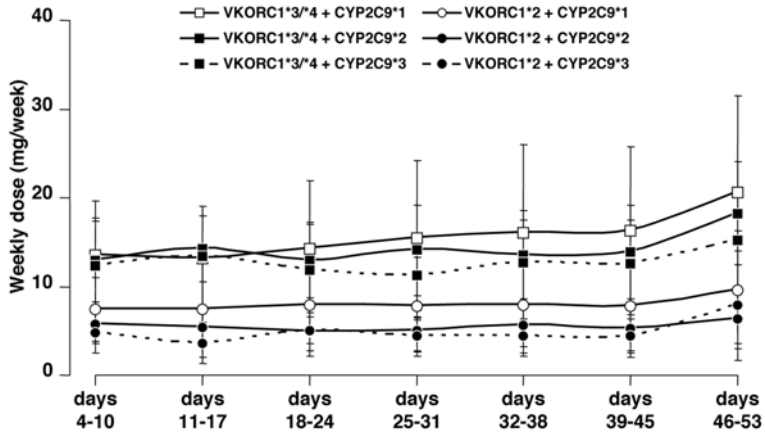

Figure 2. Mean acenocoumarol doses (mg/week, ± SD) required by patients with different genotype combinations during the first 7 weeks of therapy

Among carriers of VKORC1*2, associated with the lowest mean weekly dose requirement, the presence of the CYP2C9*2 variant allele decreased the mean dose required during the first weeks of therapy compared to carriers of CYP2C9*1. The extent of the reduction ranged from 18% (1.7 mg, 95%CI of diff: -1.48 – 4.89) during the 1st week of therapy to 27% (3.3 mg, 95%CI of diff: -1.95 – 8.51) during the 7th week of therapy. The presence of the CYP2C9*3 variant allele also decreased the required mean weekly dose compared to CYP2C9*1. The decrease

ranged from 25% (2.6 mg, 95%CI of diff: -2.25 – 7.35) during the 1st week of therapy to 14% (1.8 mg, 95%CI of diff: -11.2 – 14.78) during the 7th week of therapy.

Among carriers of VKORC1*3 or VKORC1*4, associated with the highest mean weekly dose requirement, the presence of the CYP2C9*2, compared to carriers of CYP2C9*1, decreased the dose requirement from 3.4% (0.6 mg, 95%CI of diff: -7.25 – 8.46) during the 1st week of therapy to 12% (2.4 mg, 95%CI of diff: -10.68 – 15.43) during the 7th week of therapy. The presence of the CYP2C9*3 variant allele also decreased the mean weekly dose required from 7.4% (2.6 mg, 95%CI of diff: -6.98 – 9.33) during the 1st week of therapy to 23% (5.4 mg, 95%CI of diff: -6.48 – 17.2) during the 7th week of therapy.

In all, carriers of both the VKORC1*3 or *4 allele and the CYP2C9*1 wild-type allele had the lowest mean INR on day 4 of therapy and required the highest mean weekly dose during the first weeks of therapy (Figure 1 and 2), while carriers of both VKORC1*2 and CYP2C9*3 alleles had the highest mean INR on day 4 and required the lowest mean weekly dose during the first weeks of therapy. Compared to the former, they had roughly 3 times higher mean INR [5.9 *vs* 2.2; 95%CI of diff.: 1.48 – 5.99], and required an average 55% lower mean weekly dose (~ 10.5 mg) of acenocoumarol [95%CI of diff.: 2.89 – 14.545 at week 1; -0.83 – 26.38 at week 7].

Effect of CYP2C9 and VKORC1 combinations on the percentage of time spent within, below or above the INR target range

The mean percentages of time spent within, above and below the INR target range were evaluated for each genotype combination. Table 6 shows that carriers of all the combinations spent approximately 50% of the initial phase of treatment in the

target range. However, among carriers of VKORC1*2, carriers of the CYP2C9*3 variant allele spent a higher percentage of time above their target range than carriers of CYP2C9*1 allele and a smaller percentage of time below it. Among carriers of VKORC1*3 or *4, the presence of the CYP2C9*2 or CYP2C9*3 variant alleles had the same effect, increasing the mean percentage of time spent above their target range compared to carriers of the CYP2C9*1 allele and decreasing the percentage of time spent below it.

Table 6. Mean and range of the percentage of time spent in, below or above the INR target range by carriers of different *VKORC1* and *CYP2C9* genotype combinations. The means, the p-value and 95% confidence intervals (CI) were evaluated taking as references the VKORC1*2 + CYP2C9*1 and the VKORC1*3 or *4 + CYP2C9*1 genotype combinations, respectively.

		% days spent in the INR target range	**% days spent below the INR target range**	**% days spent above the INR target range**
VKORC1*2 + CYP2C9*1 n=31	Mean	48.8	19.0	32.3
	Range	20 - 92	0 - 62	0 - 73
	p; [95%CI]	ref	ref	ref
VKORC1*2 + CYP2C9*2 n=11	Mean	47.7	22.6	29.7
	Range	20 - 70	3 – 58	0 – 74
	p; [95%CI]	1.00; [-18.82 – 21.03]	1.0; [-25.79 – 18.48]	1.0 [-21.98 – 27.09]
VKORC1*2 + CYP2C9*3 n=6	Mean	49.1	9.0	41.9
	Range	15 - 85	0 - 25	5 - 85
	p; [95%CI]	1.00 [-41.98 – 41.35]	0.67 [-9.87 – 29.78]	1.00 [-40.83 – 21.54]
VKORC1*3 or *4 + CYP2C9*1 n=19	Mean	50.0	41.5	8.5
	Range	0 – 85	0 - 100	0 - 50
	p; [95%CI]	ref	ref	ref
VKORC1*3 or *4 + CYP2C9*2 n=9	Mean	58.3	21.0	20.8
	Range	4 - 100	0 - 62	0 - 96
	p; [95%CI]	0.99 [-45.74 – 29.31]	0.4 [-10.42 – 51.47]	1.0 [-40.60 – 15.98]
VKORC1*3 or *4 + CYP2C9*3 n=7	Mean (SD)	54.9	24.6	20.4
	Range	38 - 78	3 - 58	0 - 51
	p; [95%CI]	1.0 [-27.72 – 17.90]	0.67 [-15.09 – 48.80]	1.0 [-42.86 – 18.96]

Finally, compared to carriers of the VKORC1*3 or *4 plus CYP2C9*1 alleles combination, a lower percentage of time spent below the target range was found in carriers of the VKORC1*2 plus CYP2C9*3 combination (9.0%, range: 0-25 vs 41.5, range: 1-100; 95%CI of diff: 8.4 – 56.6). They also spent a higher percentage of time above the target range [41.9%; range:5–85 vs 8.5, range: 0-50; 95%CI of diff: 0.70 – 66.12], in accordance with the correspondingly lower acenocoumarol doses required during the same period and the higher mean INR on day 4.

Exclusion of patients taking concomitant medications known to increase (n= 44) or decrease (n= 17) the INR did not significantly change the observed effect of *VKORC1* and *CYP2C9*. The outcomes of the effect of the *VKORC1* and *CYP2C9* genotype combinations on dose requirement, as well as their effect on the first INR value, the risk of severe over-anticoagulation and the percentage of time spent within, below or above the INR target range remained the same, with no loss of statistical significance. The results of the single *CYP2C9* variant alleles and *VKORC1* haplotypes on the risk of severe over-anticoagulation also remained the same. For the outcomes of the effect of single *CYP2C9* variant alleles on dose requirement, statistical significance for the difference between carriers of at least one CYP2C9*3 variant allele compared to carriers of only wild-type allele was lost (95%CI: -1.09 – 6.23 at week 1; 95%CI: -0.17 – 1.09 at week 7). For the outcomes of the effect of single *VKORC1* haplotypes on the first INR value, significance for the difference between carriers of the homozygous VKORC1*4/*4 haplotype compared to carriers of the homozygous VKORC1*2/*2 haplotype was lost (95%CI: -0.17 – 4.28). The same occurred for carriers of the heterozygous VKORC1*2/*4 haplotypes (95%CI: -0.60 – 3.33).

Discussion

This study was planned and performed to evaluate the effect of all the common gene variants, previously identified as modifiers of oral anticoagulant therapy with vit.K antagonists, on the initial phase of administration of acenocoumarol, a drug that was studied so far less than warfarin in terms of pharmacogenetics. The initial phase of treatment was chosen as the most critical and unstable, thereby engendering a risk of both over-anticoagulation (and hence of bleeding) and under-coagulation (and of thrombosis).

The first finding of this study is that, pertaining to the effect of the *CYP2C9* gene, only the CYP2C9*3 variant allele plays an important modifying role during the first 7 weeks of treatment, because its presence significantly reduces the required dose of acenocoumarol and increases the risk of over-anticoagulation (INR>6) on day 4 of treatment, particularly for homozygotes[12-17]. This result is in agreement with Bodin *et al*., [28] and Schalekamp *et al.,*[29], the latter being the only study that analysed the effect of both *VKORC1* and *CYP2C9* genotypes during the initial phase of acenocoumarol treatment. However, our finding is in contrast with Gonzàlez-Conejero *et al.,* [30], who reported that *CYP2C9* polymorphisms have no effect on the first INR value after 3 days of acenocoumarol therapy. This discrepancy might be due to the fact that Gonzàlez-Conejero *et al.,* performed the analysis combining together CYP2C9*2 and CYP2C9*1 carriers.

Pertaining to the *VKORC1* gene, the presence of the ancestral VKORC1*1 haplotype [22] was found at a low frequency in our Italian population, in contrast with Geisen *et al.,* [22] and Osman *et al*., [38], who showed that this haplotype is absent in European populations. Interestingly, a rare *VKORC1* mutation, p.D36Y, previously identified in individuals who required an average warfarin dose greater than 10 mg/day [39], was recently reported to tag a unique haplotype found on a VKORC1*1 background in Ashkenazi and Sephardi Jews [40]. Replication studies

on other Caucasian populations are thus required to establish the real frequency of this allele.

Homozygotes for the VKORC1*2 haplotype required the lowest acenocoumarol doses and had a 4.5 times increased risk of over-anticoagulation (INR>6) on day 4 compared to homozygotes for the VKORC1*3 or VKORC1*4 haplotypes. Both these haplotypes had a similar effect on INR and dose requirements, being associated with higher acenocoumarol doses and lower INR on day 4. This confirms the findings of Osman *et al.,* [38] in warfarin-treated patients. The VKORC1*2 haplotype is characterized by the presence of the c.173+1000 C/T polymorphism, which is in complete linkage disequilibrium with the promoter polymorphism c.-1639G/A, that re-establishes a E-box consensus sequence reducing the promoter activity and thus *VKORC1* mRNA expression [41]. The VKORC1*3 haplotype is characterized by the presence of the c.492+134 G/A polymorphism, first identified by D'Andrea *et al.,* [35] in Italian patients on warfarin therapy, and then described by Geisen *at al.* [22] to be associated with a partial resistence to warfarin. As expected from the effect of single alleles, the highest dose requirement and the lowest INR on day 4 were found for the homozygous VKORC1*3 or *4 plus CYP2C9*1 genotype combination, whereas the lowest doses, the highest INR as well as an increased risk of over-anticoagulation were for the VKORC1*2 plus CYP2C9*3 genotype combination. Moreover, in the initial phase of therapy patients carrying all the investigated *CYP2C9* and *VKORC1* combinations spent the same amount of time in the INR therapeutic range. Carriers of VKORC1*3 or *4 plus CYP2C9*1, associated with a greater resistance to acenocoumarol, spent a large percentage of the remaining time below the target range, whereas carriers of the VKORC1*2 plus CYP2C9*3 combination, associated with a higher sensitivity to the drug, spent a greater amount of the remaining time above the target range. This is in agreement with Schalekamp

et al., [29], who reported that only carriers of a combination of *CYP2C9* and VKORC1 C1173T polymorphisms have a high risk of over-anticoagulation.

Therefore, based on the obtained results we could conclude that it may be necessary to test not only the *CYP2C9* genotype (particularly the CYP2C9*3 variant allele) and the *VKORC1* c.173+1000 C/T polymorphism (VKORC1*2 haplotype), but also the *VKORC1* c.492+134 G/A (VKORC1*3) and the c.173+525 C/T (VKORC1*4) to identify patients with the extreme phenotypes: those with a higher sensitivity to acenocoumarol and more exposed to the risk of over-anticoagulation (bleeding); those with a greater resistance to the drug are more exposed to the risk of under-anticoagulation (thrombosis), particularly in the initial phase of treatment.

To our knowledge this is the first work that take into consideration the effect of the VKORC1*3 and VKORC1*4 haplotypes other than that of VKORC1*2 in the initial phase of acenocoumarol therapy.

The *CYP2C9* and the *VKORC1* genotypes, together with age, explain 26% of the observed INR variability. They also account -- together with sex, BSA, age and indications for non-ischemic cardiac disease or venous thrombosis -- for 27% of the variability in dose requirement observed on the 7th week of therapy. In both situations, our results confirm that a major role is exerted by *VKORC1*, followed by *CYP2C9* genotype [23, 28-29]. The variability explained in this study is lower that the variability explained in the warfarin response [42]. These differences might be due to different study design and all variants included in the model, as well as to other unknown genetic and non-genetic variables not considered in this study.

Our study also included also some patients treated with other medications known to interfere with the effect of the anticoagulant drug, with the possibility to affect the results and this seems to be a limit of the study design. However, the regression analysis adjusted for these risk factors did not change the effect of the

CYP2C9 and *VKORC1* genotypes. The same study design was also used in previous reported studies [15, 29, 31, 43-45]. Moreover, the effect of *CYP2C9* and *VKORC1* genotypes was also confirmed after the exclusion of these patients from the analysis.

A limit of the study is that our cohort includes also some patients treated with other medications known to interfere with the effect of the anticoagulant drug, with the possibility to affect the results. However, the same study design was used in other studies [15, 29, 31, 43-45] and the effect of *CYP2C9* and *VKORC1* genotypes was also confirmed after the exclusion of these patients from the analysis.

In conclusion, both the detection of the VKORC1*2, *3 and *4 haplotypes as well as the CYP2C9*3 variant allele might be useful to select not only the most sensitive patients, exposed to a higher risk of over-anticoagulation, but also the most resistant ones, exposed to the risk of thrombosis recurrence. On August 2007, the USA Federal Drug Administration updated the label of warfarin to include information on pharmacogenetic testing and to encourage the use of this information in dosing individual patients initiating warfarin therapy [46]. Up to now, several dosing pharmacogenetic algorithms to predict *a priori* the maintenance coumarin dose [23-25, 47-53], as well as a new dose-refinement nomogram to guide clinicians in adjusting warfarin dose [54] have been developed. A randomized study of personalized, pharmacogenetic-guided warfarin dosing using both *CYP2C9* and *VKORC1* variants plus clinical factors in 200 patients was recently published [55], showing an improvement in the accuracy and efficiency of warfarin dose initiation, particularly for wild-type and carriers of multiple variant genotypes. Despite this, a reduction in out-of –range INRs was not achieved.

Future perspective

Pharmacogenetics results can be useful to identify patients more sensitive to coumarin drugs, and thus exposed to a higher risk of over-anticoagulation, as well as those who are more resistant to anticoagulation, and thus exposed to the risk of recurrence of thrombosis. The improvement and validation, by means of prospective clinical trials, of current pharmacogenetic-guided coumarin dosing algorithms, might help to predict the dose requirement of anticoagulant of coumarin type in order to aid clinicians to initiate therapy avoiding adverse events. This issue will be tackled by a prospective double-blind, randomized three-arm trial on 1.965 participants, sponsored by the National Heart, Lung and Blood Institute and meant to compare three possible approaches to guiding warfarin therapy initiation: a strategy based on an algorithm using both clinical and genotypic information on genetic variants in at least CYP2C9 and VKORC1 genes, a strategy based on an algorithm using only clinical information and a standard, guidelinebased strategy [56].

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