Chapter 6

Variant VKORC1 and CYP2C9 alleles in patients with diffuse alveolar haemorrhage caused by oral anticoagulants

P Wijnen, C Linssen, G Haenen, O Bekers, M Drent

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Abstract

Background
Diffuse alveolar hemorrhage (DAH) is a life threatening bleeding complication that can occur as a result of oral anti-coagulation therapy.
We hypothesized that in patients treated with coumarins, alveolar hemorrhage is associated with vitamin K epoxide reductase (*VKORC1*) and cytochrome P450 (CYP) 2C9 *(CYP2C9)* variant alleles. In addition, in the case of acenocoumarol use, *CYP2C19* allelic variants also play a role.

Study design
During a 7-year period patients using coumarins with confirmed DAH were gathered. Out of 173 confirmed DAH cases, 75 received oral anticoagulants, and 63 patients (84%) of these 75 were included because their DNA was available. For genotyping the *CYP2C9**2 (C430T), *CYP2C9**3 (A1075C), *CYP2C19**2 (G681A), *CYP2C19**3 (G636A), *VKORC1* (G-1639A) and *VKORC1* (C1173T) single nucleotide polymorphisms (SNPs), real-time PCR's were performed.

Results
In 62 (98.4%) out of 63 DAH patients, variant genes were found. In 51 (81.0%) of the 63 patients *VKORC1* allelic variants (20 homozygote and 31 heterozygote) were present. In 31 (49.2%) of the 63 DAH cases *CYP2C9* variant alleles (three homozygote, 26 heterozygote, and two compound heterozygote) and in 20 (32.0%) of the 63 patients both allelic variants were observed.

Conclusion
Genotyping of four SNPs for *VKORC1* and *CYP2C9* polymorphisms is useful in predicting a high probability of the occurrence of DAH, in patients on oral anticoagulants. Early and timely use of genotyping is recommended to prevent a fatal outcome and to provide a safer and more individualized anticoagulant therapy.

Background

Coumarin-based oral anticoagulants act as vitamin K antagonists. They are the most commonly prescribed drugs for therapy (such as in venous thrombosis or pulmonary embolism) or prophylaxis (as in chronic atrial fibrillation, prosthetic heart valves and other cardiovascular diseases) of thromboembolic conditions. The primary goal of coumarin administration is to prevent clot formation and its expansion while carefully avoiding unintended adverse drug reactions (ADR) from over-anticoagulation.[1] The effect of the therapy is monitored by the prothrombin-time international normalized ratio (INR). An INR of less than 2.0 is associated with an increased risk of thromboembolism, and an INR of 4.0 or more denotes an increased risk of bleeding.[2]

One of the bleeding complications occurring in patients receiving coumarins, is diffuse alveolar hemorrhage (DAH).[3] DAH may be fulminant and lead to death. Due to the severe effects of overdosing and the narrow therapeutic window, correct management of coumarins is challenging. A safe and effective dose has to be determined during the early phase of therapy, and maintenance doses need to be adjusted to compensate for changes in patients' weight, diet, disease state and concomitant use of other medication.[4] The challenge is becoming even more demanding because of the increased use of coumarins that is a consequence of the aging of populations in industrialised countries.

Despite the ability to closely monitor the therapeutic effect of coumarins, by means of the INR, there is a relatively high incidence of complications.[1] Since early treatment of these complications is life-saving and may result in complete recovery. Therefore, early diagnosis can be critical. At present the diagnosis of DAH is often made by an increased percentage of siderophages (>20%) in bronchoalveolar lavage fluid (BALF), indicated with Perl's staining.[5]

Instead of early diagnosis, prevention would of course be much more preferable. The relatively high inter-individual drug requirement indicates that genetic factors may impact the therapeutic effect of coumarins. The strongest predictors of coumarin induced anticoagulant effects appear to be genes encoding for the enzyme vitamin K epoxide reductase complex 1 (VKORC1), the target of vitamin K antagonists. The enzyme VKORC1 recycles vitamin K epoxide to the reduced form of vitamin K, an essential cofactor in the formation of active vitamin K dependent clotting factors II (prothrombin), VII, IX, and X through γ-glutamyl carboxylation, see also Figure 6.1.[6]

Another predictor appears to be cytochrome P450 (CYP) 2C9, the enzyme mainly responsible for the metabolism of coumarins.[4,7,8] For instance patients with the common, functionally defective, *2 and *3 allelic variants of the *CYP2C9* gene require significantly lower maintenance doses, take longer to achieve dose stabilization, and are at higher risk for serious and life-threatening bleeding than are patients without these variants.[8]

When using acenocoumarol as an oral anticoagulant, one might even consider CYP2C19, although its contribution to the metabolism of acenocoumarol is small compared with CYP2C9.[9]

This study evaluates the association between the occurrence of DAH in patients after initiating coumarin anticoagulant therapy and the presence of *VKORC1* and *CYP2C9* allelic variants.

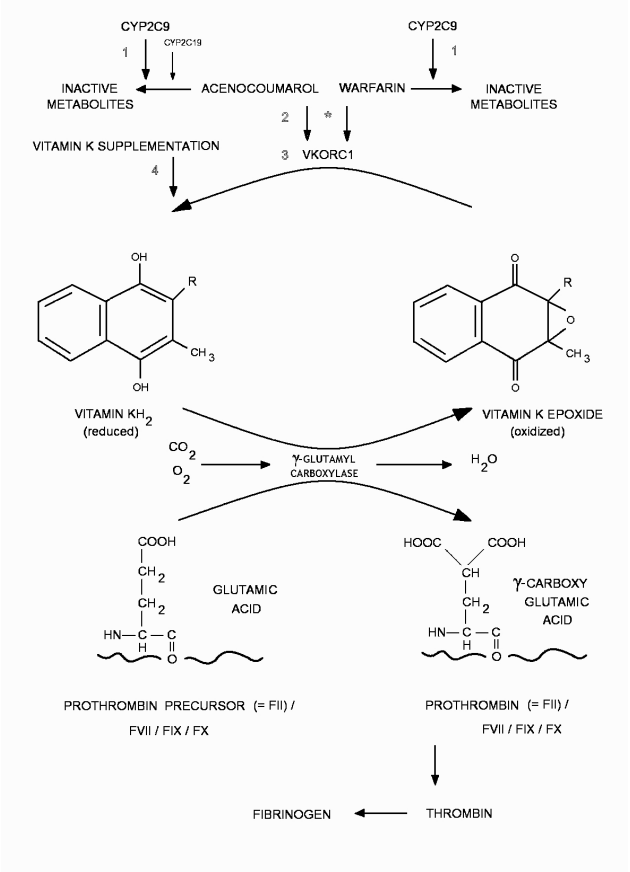

Figure 6.1 Interactions in the vitamin K cycle and coagulation. The vitamin K cycle plays an important role in the formation of functional vitamin K-dependent clotting factors (FII, FVII, FIX, and FX). Within the vitamin K cycle VKORC1 is responsible for the reduction of vitamin K epoxide (the inactive form) to vitamin KH_2 (the active form) and the target for oral anticoagulants. Interactions can occur on several levels: (1) If variant *CYP2C9* alleles are present, inadequate metabolism of coumarins by the affected CYP enzyme will result in more inhibition of VKORC1. (2) If variant *VKORC1* alleles are present, the VKORC1 enzyme will be more sensitive to inhibition by anticoagulants, resulting in over-anticoagulation. (3) Inhibition of VKORC1 by coumarins will prevent vitamin K epoxide to revert back to vitamin KH_2, slowing down the vitamin K cycle. This inhibits the formation of vitamin K-dependent clotting factors. High levels of coumarins cause over-anticoagulation. (4) Vitamin K supplementation stimulates the vitamin K cycle, thus preventing over-anticoagulation. * indicates antagonism.

Materials and methods

Setting and study population

Patients who were diagnosed with DAH at the Maastricht University Medical Centre (Maastricht, the Netherlands) from 2002 until 2009 were enrolled in the study. The inclusion criteria were bronchoalveolar lavage (BAL) performed in the diagnostic work-up and confirmed anticoagulant therapy initiated before the clinical episode of DAH. During this 7-year period, 1258 BAL analyses were carried out, and 252 cases with suspected DAH were identified. BAL was performed according to the hospital protocol, as reported previously.[10] A total of 200 macrophages were counted, the total number of iron-stain (Perl's stain)-positive macrophages were expressed as a percentage of the 200 cells counted. A percentage of >20% iron-positive macrophages was considered indicative for an alveolar hemorrhage.[5] Of the obtained BALF samples, 173 samples had >20% iron-stain-positive cells. In 75 of these 173 confirmed DAH cases, treatment with coumarins had been recently initiated. This study was a retrospective evaluation, and DNA was available only in 63 (84%) out of 75 cases. In 40 of these 63 DAH cases, either genotyping for *CYP2D6*, *CYP2C9*, and *CYP2C19* had been performed previously to evaluate whether there might be a drug-induced reaction involved in the observed clinical deterioration, or EDTA material was still available. In 23 cases, DNA was isolated from the cells in the BALF samples. All remaining samples were genotyped for CYP polymorphisms. In addition, for this study, *VKORC1* genotyping was performed in these 63 DAH cases.

A control population of 173 healthy, unrelated, Caucasian volunteers was also genotyped for the studied single nucleotide polymorphisms (SNPs).

The study was performed in accordance with the Declaration of Helsinki and its amendments. Written informed consent was obtained. The protocol was approved by the Medical Ethics Board of the Maastricht University Medical Centre.

Collection of clinical data

Inpatient and outpatient medical records of these 63 unrelated patients of Caucasian origin, presenting with DAH and using coumarins, were reviewed. Two patients received phenprocoumol, and the remaining 61 patients received acenocoumarol. Routine laboratory tests, chest X-rays, and high-resolution CT scans were reviewed in all patients. Appropriate and relevant biopsies were also evaluated when available (18 cases).

Genotyping

In addition to the previously determined polymorphisms, *VKORC1* genotyping was performed.
DNA was obtained from all subjects by using venous EDTA anticoagulated blood or BALF samples and isolated with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.
For genotyping the *CYP2C9**2 (C430T), *CYP2C9**3 (A1075C), *CYP2C19**2 (G681A), *CYP2C19**3 (G636A), *VKORC1* (G-1639A), and *VKORC1* (C1173T) SNPs, three real-time PCR Fluorescence Resonance Energy Transfer (FRET) analyses were performed. FRET LightMix® assays (cat.-no. 40-0298-16, 40-0304-16, and 40-0302-16; TIB MOLBIOL, Berlin, Germany) on the LightCycler® (Roche Diagnostics) were used, according to the manufacturer's protocols. These three FRET assays simultaneously determined the two SNPs of *CYP2C9*, *CYP2C19* and *VKORC1*, respectively, in separate capillaries. Each assay consisted of a duplex reaction measuring the melting curves of the used specific fluorescent probes in two different channels, each with a distinct wavelength. Positive (heterozygote, provided with the kit) and negative controls were determined with each run.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0 software for Windows (SPSS Inc., Chicago, IL, USA) for Windows. The chi-square test was used to test for statistically significant differences between groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were derived from these tables to evaluate the strength of associations between genotypes and the DAH event. Actual allele distributions were compared against the expected frequencies that were calculated, using the Hardy–Weinberg equilibrium. Deviations from Hardy–Weinberg equilibrium were analysed using the chi-square test. A p-value of <0.05 (two-sided) was considered to indicate statistical significance. A Bonferroni correction was applied, if appropriate, to adjust for multiple comparisons ($p<0.01$, indicating statistical significance).

Results

The characteristics and summary of relevant clinical data of the studied patients (15 females and 48 males) with DAH are listed in Table 6.1. Data subdivided on the basis of the presence of the studied polymorphisms did not show substantial differences (Table 6.1).

Table 6.1 Demographic and clinical characteristics of the patients (n=63) with diffuse alveolar hemorrhage[a].

Genotype group	Sex (M/F)	Age (y)	Hb (mmol/l) M (8.2-11.0) F (7.3-9.7)	Ht (l/l) M (0.41-0.52) F (0.36-0.48)	Thrombocytes (x 10^9/l) [130-350]	LD (U/l) [0-480]	ANCA (neg/ND)	ANF (neg/ND)	Deceased (yes/no)
Total population	48/15	63.1±15.9 (20-85)	6.7±1.3 (4.0-9.4)	0.33±0.06 (0.20-0.46)	283±156 (14-971)	937±1237 (319-10170)	38/24 (1 pos)	38/24 (1 pos)	37/26
VKORC1 and *CYP2C9* variant	14/6	63.9±18.5 (20-85)	7.0±1.5 (4.1-9.4)	0.35±0.07 (0.20-0.46)	268±136 (14-669)	768±419 (319-1846)	11/8 (1 pos)	11/8 (1 pos)	10/10
VKORC1 variant only									
AA/TT	9/3	58.8±14.9 (35-77)	5.9±1.6 (4.0-9.1)	0.29±0.08 (0.21-0.45)	224±112 (81-459)	1614±2725 (396-10170)	8/4	9/3	8/4
GA/CT	15/4	65.6±13.7 (27-82)	6.7±1.1 (4.8-8.8)	0.33±0.05 (0.23-0.43)	303±190 (104-971)	788±359 (389-1778)	9/10	9/10	12/7
CYP2C9 variant only	9/2	61.6±16.9 (34-80)	6.8±0.9 (5.3-7.8)	0.34±0.05 (0.27-0.40)	354±151 (116-571)	798±254 (418-1139)	10/1	9/2	6/5
No variant alleles	1/0	66.0	6.3	0.31	129	542	0/1	0/1	1/0

[a] Data are presented as absolute number or means±SDs, with ranges in parentheses if appropriate.
AA/TT=homozygote variants *VKORC1* -G1639A and C1173T; ANCA=anti-neutrophil cytoplasmic antibody; ANF=antinuclear factor; F=female; GA/CT=heterozygote variants *VKORC1* -G1639A and C1173T; Hb=hemoglobin; Ht=hematocrit; LD=lactate dehydrogenase; M=male; ND=not done; neg=negative; pos=positive.

The reasons for the patients being on anticoagulants were as follows: atrial fibrillation or flutter (n=34); previous myocardial infarction (n=12); chronic heart failure (n=7); lung embolisms (n=4); valve replacement surgery (n=4); deep-vein thrombosis (n=2). The average dose of the coumarins was low (maximum 2 mg/day, with an initial dosing scheme of 6 mg/day for one day, then 4 mg/day for one day, and then 2 mg/day or 4 mg/day for two days and then 2 mg/day in elderly patients). The INR was above the therapeutic range within two weeks after initiation of anticoagulant treatment in all DAH cases (median 5.50, with 80% INR >4.00 and 40% INR >6.00). Most of the patients had several episodes of an increased INR during the follow-up, were difficult to normalize, and had to be kept on lower anticoagulant doses than is standard for the general population. Immunological analysis revealed no abnormalities, and no underlying systemic diseases were found. The high-resolution CT showed wide spread signs of DAH, with patchy bilateral or diffuse areas of ground-glass attenuation in all cases. A BAL was performed in all subjects, showing hemorrhagic BALF with a markedly positive iron staining (>22%, mean 59.3±25.7) and the presence of erythrocytes and pneumocytes type II, confirming the diagnosis DAH.[10]

The allele frequencies and genotype distribution in the DAH patients were determined and compared with the distribution of the same polymorphisms in a healthy, unrelated, Caucasian population from our hospital and from populations in the literature (Table 6.2).[11,12] Allele frequencies and genotype distributions of both control populations were in Hardy-Weinberg equilibrium. A *VKORC1* variant allele was found in 51 of the 63 patients with DAH (81.0%, $p<0.025$). This included 20 homozygotes (AA/TT), including the only two patients in our population receiving phenprocoumol, and 31 heterozygotes (GA/CT). A *CYP2C9* allelic variant was found in 31 of the 63 patients (49.2%, $p<0.025$), including three homozygotes, 26 heterozygotes, and two compound heterozygotes. Twenty (32.0%) out of the 63 DAH cases had both *VKORC1* and *CYP2C9* allelic variants. In 31 (49.2%) out of these 63 DAH cases, only a *VKORC1* allelic variant was found. Of the 12 DAH cases without a *VKORC1* allelic variant (GG/CC), five were *CYP2C9**1/*2 heterozygotes, four were *CYP2C9**1/*3 heterozygotes, and two had a compound heterozygous variant *CYP2C9**2/*3 genotype. The remaining subject had no variant of the studied alleles. When comparing patients with controls for the presence of a polymorphism (polymorphism present versus no polymorphism), a significant difference was found (OR=14.6, 95% CI: 1.96-109.3; $p<0.001$). Furthermore, a *CYP2C19* variant allele was found in one third of the DAH cases. In all of these patients, this coincided with the presence of a *CYP2C9* and/or *VKORC1* allelic variant (Table 6.3).

Table 6.2 Allele frequencies and polymorphism distribution in diffuse alveolar hemorrhage (DAH) patients compared with healthy volunteers and historical controls.

	Patients with DAH[a]			Healthy volunteers[b]			Controls from the literature[c]		
	CYP2C9 (%)	*VKORC1* (%)	*CYP2C19* (%)	*CYP2C9* (%)	*VKORC1* (%)	*CYP2C19* (%)	*CYP2C9*[11] (%)	*VKORC1*[11] (%)	*CYP2C19*[12] (%)
No variant allele	50.8	19.0	66.7	61.9	29.5	76.3	64.0	34.0	75.3
Variant allele	49.2[d]	81.0[e]	33.3[f]	38.1	70.5	23.7	36.0	66.0	24.7
Allele[g]									
*1	71.4		81.7	79.7		86.4	80.0		86.5
*2	15.9		18.3	13.9		13.6	13.5		13.3
*3	12.7		0.0	6.4		0.0	6.5		0.2
G/C		43.7			52.3			58.5	
A/T		56.3			47.7			41.5	

[a] n=63; sex 76.2% male, 23.8% female; age range=20–85 y. [b] n=173; sex 56.6% male, 43.4% female; age range=19–59 y. [c] For *CYP2C9* and *VKORC1*: n=200; sex 50% male, 50% female; age range=18–24 y; For *CYP2C19*: n=736; sex 82% male, 18% female; age range=18–79 y. [d] p=0.022 vs healthy volunteers; p=0.006 vs historical controls. [e] p=0.021 vs healthy volunteers; p=0.0015 vs historical controls. [f] p=0.024 vs healthy volunteers; p=0.046 vs historical controls. [g] For *CYP2C9*: *1=wild type, *2=430T, and *3=1075C; for *CYP2C19*: *1=wild type, *2=681A and *3=636A. *VKORC1* SNPs are G-1639A and C1173T; genotype G/C is wild type and A/T is variant.

Table 6.3 Influences on the coagulation: allelic variants and co-medication in patients (n=63) with diffuse alveolar hemorrhage.

Patient no.	*CYP2C9*[a]		*VKORC1*[b]		*CYP2C19*[c]		Influence of co-medication
	genotype	influence	genotype	influence	genotype	influence	(yes/no)
3	*1/*3	Yes	AA/TT	Yes	*1/*2	Yes	2/1
1	*1/*2	Yes	AA/TT	Yes	*1/*2	Yes	1/0
1	*2/*2	Yes	AA/TT	Yes	*1/*1	No	0/1
1	*1/*3	Yes	AA/TT	Yes	*1/*1	No	1/0
2	*1/*2	Yes	AA/TT	Yes	*1/*1	No	2/0
2	*1/*1	No	AA/TT	Yes	*2/*2	Yes	1/1
3	*1/*1	No	AA/TT	Yes	*1/*2	Yes	2/1
7	*1/*1	No	AA/TT	Yes	*1/*1	No	3/4
2	*2/*2	Yes	GA/CT	Yes	*1/*1	No	1/1
6	*1/*3	Yes	GA/CT	Yes	*1/*1	No	5/1
4	*1/*2	Yes	GA/CT	Yes	*1/*1	No	3/1
11	*1/*1	No	GA/CT	Yes	*1/*2	Yes	8/3
8	*1/*1	No	GA/CT	Yes	*1/*1	No	4/4
2	*2/*3	Yes	GG/CC	No	*1/*1	No	1/1
1	*1/*3	Yes	GG/CC	No	*1/*2	Yes	0/1
3	*1/*3	Yes	GG/CC	No	*1/*1	No	2/1
5	*1/*2	Yes	GG/CC	No	*1/*1	No	2/3
1	*1/*1	No	GG/CC	No	*1/*1	No	1/0

[a] *CYP2C9* SNPs are C430T and A1075C; allele designations: *1=wild type, *2=430T, and *3=1075C. [b] *VKORC1* SNPs are -G1639A and C1173T; genotype GG/CC is homozygous wild type and AA/TT is homozygous variant. [c] *CYP2C19* SNPs are G681A and G636A; allele designations: *1=wild type, *2=681A and *3=636A.

The influence of allelic variants and co-medication on the anticoagulation in each individual case is summarized in Table 6.3. In about 60% of all the patients, co-medication was prescribed that might have influenced the coagulation. In only one patient - the one without any variant alleles in the *VKORC1* or *CYP2C9* - the co-medication might have caused the bleeding. In this patient, a drug-drug interaction was most likely, with no fewer than four drugs (amiodarone, paroxetine, pantoprazole, and clopidogrel), being used that could have interacted with the anticoagulant. The drugs prescribed to the patients that might have influenced the anticoagulation are listed in Table 6.4.
Since May 2007, genotyping for the *VKORC1* polymorphism, together with the earlier described CYP polymorphisms, has been available on request for clinicians in our hospital. Subsequently, in the period between May 2007 and December 2008, 11 patients with DAH were identified and included in this study. In all of these patients, vitamin K supplementation (1 mg/day, orally) was started. Out of the 11 patients, 10 responded quite well, recovered, and are still alive. Of the 63 studied DAH cases, 37 patients died, primarily because of complications related to heart failure in combination with DAH.

Table 6.4 Medication influencing the coagulation in the patients in which this was relevant (n=39).

Administered co-medication	No. of patients
amiodarone	15
amitriptyline	1
aspirin	5
atorvastatine	7
carvedilol	5
clopidogrel	2
colchicine	1
esomeprazol	1
felodipine	1
fluoxetine	1
insulin	2
isoniazid	1
levothyroxine	1
nifedipine	1
omeprazole	5
pantoprazole	15
paroxetine	4
prednisone	8
ranitidine	1
rifampin	1
simvastatin	2
trimethoprim/sulfamethoxazole	2
valproic acid	2
verapamil	2

Discussion

Anticoagulants can cause fatal pulmonary hemorrhage. Barnett et al.[13] reported a case of DAH due to superwarfarin ingestion. More recently, Erdogan et al.[3] reported a case of DAH associated with coumarin therapy. We described a case of DAH in a patient who had malnutrition and was taking antibacterials and anticoagulants; at that time, genotyping was not yet available.[14] DAH results in accumulation of iron in the lungs and, in turn, iron causes oxidative stress and inflammation. It has been suggested that oxidative damage plays a role in the pathophysiology of various diseases.[15] It is important to prevent or recognize DAH at an early stage to avoid irreversible damage. Particularly, in critically ill patients with unexplained infiltrates, DAH should be considered. DAH events can occur as a result of over-anticoagulation due to coumarin sensitivity, caused by *VKORC1* or *CYP2C9* polymorphisms, resulting in a relative vitamin K deficiency. Prophylactic administration of vitamin K to patients at risk can prevent severe damage.[16-18] Just recently, the information gathered from genotyping in this study has become available to clinicians. Subsequently, vitamin K supplementation (1 mg/day) was initiated in 11 DAH cases, resulting in a stabilization of the INR and a positive outcome in 10 of these 11 DAH cases. This supports the concept that the use of new pharmacogenetic-based dosing schemes and the concomitant application of low-dose vitamin K with coumarins will greatly improve coumarin drug safety.[18,19]

Pharmacogenomics uses the tools of human genetics to tailor medical treatment to an individual's genetic make-up. To this end, phenotypic manifestations, a therapeutic outcome, or ADRs are considered in relation to the underlying genetic background of a patient.[7,20] The identification of the molecular target of coumarins, VKORC1, has greatly improved the understanding of coumarin treatment and illuminated new perspectives for a safer and more individualized oral anticoagulation therapy. Rieder et al.[4] previously demonstrated that the *VKORC1* genotype appeared to be the most important genetic factor determining variability in coumarin dose; its effect was approximately three times higher than that of the *CYP2C9* genotype. More recently, in line with this, Schwarz et al.[16] concluded that the initial variability was more strongly associated with genetic variability in the pharmacogenetic target of coumarins, VKORC1, than with CYP2C9.

Variations and SNPs within the translated and non-translated regions of the *VKORC1* gene have been shown to cause coumarin resistance and sensitivity, respectively.[21] A frequent SNP within the *VKORC1* promoter (G-1639A) has been identified as a major determinant of coumarin sensitivity, reducing vitamin K epoxide reductase enzyme activity to 50% of wild type (GG=fully functional). Homozygous carriers of the *VKORC1* -1639A allele (AA) are

strongly predisposed to coumarin sensitivity and require lower coumarin dosages. However, the link between DAH and the presence of *VKORC1* and *CYP2C9* variant alleles has never been made before.

To the best of our knowledge, our study is the first one evaluating the association between the occurrence of a serious adverse reaction of anticoagulant therapy, DAH, and the presence of relevant polymorphisms. We found that in 62 (98.4%) of the patients in our study population, a variant allele was present. In 81.0% (51/63) of the studied patients, the bleeding complication could be explained by the *VKORC1* haplotype (61.0% without and 39.0% with a *CYP2C9* allelic variant) alone. As also shown in Table 6.2, only 19.0% of the patients had no *VKORC1* 1173T/-1639A variant alleles, compared with 29.5% in a healthy volunteer population (n=173) and 34.0% in a historical control population (n=200) from the literature.[11] In 11 of the 12 DAH cases without a *VKORC1* variant the *CYP2C9* variant allele could explain the problems in reaching an appropriate INR. The present *CYP2C9* functionally defective allelic variant required a 34% lower maintenance dose for the *1/*3 genotype and a 61% reduction for the *2/*3 genotype, compared with 13% for the *CYP2C9**1/*2. In the one remaining patient without any variant of the alleles studied, the high INR and subsequent DAH event seemed attributable to drug-drug interactions. One third of the patients had an extremely high risk as they appeared to have both genetic risk factors that are known to stratify patients into low dose/high-risk cases.[16] Furthermore, the patients with *VKORC1* and/or *CYP2C9* allelic variants present need longer times before dose stabilization and are at a higher risk for serious and life-threatening bleeding, including DAH, than patients without these variants.[8,22]

Our observation confirms that genotyping four SNPs, namely of the *VKORC1* and the *CYP2C9* genes, predicts a high risk of overdosing with coumarins (warfarin, acenocoumarol, phenprocoumon).[23] Although other studies have reported a strong linkage disequilibrium between the SNPs in the *VKORC1* gene, our own experience shows that sometimes only one of the examined SNPs can display a variant allele.[24,25] Accordingly, this implies a potential risk factor could be missed if only one SNP is examined. Moreover, in this study, the *VKORC1* results were obtained in one run, using a reagent combining primers and probes for both SNPs, without any extra time or costs. In the case of oral anticoagulation with acenocoumarol, genotyping for the *2 (G681A) and *3 (G636A) allelic variants in the *CYP2C19* gene could be performed. Although acenocoumarol is mainly metabolized by CYP2C9 it is also partly metabolized by CYP2C19 (Figure 6.1).[9] Polymorphisms in this enzyme system could therefore present additional anticoagulation problems. In our population, however, this polymorphism was of minor importance. All of the subjects with a *CYP2C19* variant allele (33.3% of all of the patients) also displayed one or both of the other two studied polymorphisms.

One of the limitations of this study was the fact that DNA was available in only 63 of the 75 subjects who used oral anticoagulants and were diagnosed with DAH. Therefore, conclusions from this case series should be interpreted with care, and prospective studies should be conducted to evaluate the cost effectiveness of genotyping. Moreover, confirmation of our findings in other populations is mandatory. However, the fact that all but one of the included patients with DAH demonstrated at least one of the studied genetic defects makes the association highly likely. The merits of genotyping before starting treatment involving drugs such as coumarins, the effectiveness of which depends on genetic variants of *CYP2C9* and *VKORC1*, is still an area of debate between regulatory authorities and clinicians. Even though genotyping four SNPs is relatively cheap (about $US 200-250 in 2009) and needs to be performed only once in a lifetime, until its cost effectiveness is established, one could choose to only genotype patients who experience unstable INRs, in order to avoid serious complications. Nevertheless, as of August 2007, the US FDA issued a recommendation to genotype *CYP2C9* and *VKORC1* in warfarin product labeling, to optimize dosing schedules when prescribing warfarin.[24,26] Furthermore, it is tempting to speculate that by using individualized dose adaptation, a significant reduction of bleeding complications, including DAH, can be expected, especially in the initial drug-saturation phase.[4]

Conclusion

In all but one of the studied patients with DAH treated with coumarins, an association with either a *VKORC1* or a *CYP2C9* variant allele, or both, was found. Early and timely use of appropriate genotyping is important in case of coumarin treatment, because of the potential fatal outcome of over-anticoagulation and the fact that simple vitamin K supplementation can be life-saving. Therefore, in concordance with the FDA, genotyping of only four SNPs for *VKORC1* and *CYP2C9* allelic variants is recommended in order to provide a safer and more individualized anticoagulant therapy.

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