Genetic and Environmental Factors Affecting the Coumarin Anticoagulant Level

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Genetische en omgevingsfactoren die de intensiteit van de antistollingsbehandeling met cumarine anticoagulantia beïnvloeden

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Voor Famke en Fleur

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Chapter 1

General introduction

Historical perspective and present clinical use

Like many other pharmaceuticals, coumarin anticoagulants owe their discovery to the appreciation of an unexpected adverse reaction. In 1924, a previously undescribed hemorrhagic disorder was reported in cattle, that resulted from the ingestion of spoiled sweet clover silage [1]. After the cause was traced to a toxic reduction of plasma prothrombin, Campbell and Link, in 1939, identified the hemorrhagic agent as dicoumarol (bishydroxycoumarin) [2]. Since then, several hundred derivatives of coumarin have been synthesized [3]. Warfarin was found to be more potent than dicoumarol and was first introduced as a rodenticide. Application of warfarin as an anticoagulant in humans came about when it was observed through accidental poisonings and suicide attempts that, except for its depression of clotting factor activity, warfarin had no other significant biological effects in humans. Warfarin was first introduced into human anticoagulant therapy in 1941 [4]. In 1954, a large multicenter clinical trial of the American Heart Association resulted in a report of seemingly favourable responses of patients with myocardial infarction to dicoumarol. The indications for long-term therapy with coumarin anticoagulants have broadened considerably since then.

Coumarins are now prescribed for primary and secondary prevention of venous thromboembolism, for prevention of systemic embolism in patients with tissue or mechanical prosthetic heart valves or atrial fibrillation, for prevention of acute myocardial infarction in patients with peripheral arterial disease, for prevention of stroke, recurrent infarction, or death in patients with acute myocardial infarction, and for prevention of myocardial infarction in men at high risk [5]. Other drugs used for prevention or treatment of several thromboembolic diseases are unfractionated heparin or low-molecular-weight heparin (LMWH), thrombolytic and platelet inhibiting drugs, such as aspirin and glycoprotein IIb/IIIa antagonists. Heparins are often used together with coumarin anticoagulants in the acute treatment of thromboembolic disease because of their immediate anticoagulant effect. They are withdrawn once the coumarin anticoagulant is exerting its full effect. Therapy with heparins, thrombolytic and platelet inhibiting drugs are beyond the scope of this thesis and will not be discussed further.

Mechanism of action of coumarin anticoagulants

Coumarin anticoagulants decrease the synthesis of vitamin K-dependent clotting factors II, VII, IX and X by inducing a functional deficiency of reduced vitamin K [5, 6]. The normal carboxylation of factors II, VII, IX and X requires reduced vitamin K, which is usually oxidized to vitamin K epoxide in the process. Reduced vitamin K is regenerated from the vitamin K epoxide by an epoxide reductase. Coumarin anticoagulants block this enzyme and prevent the regeneration of reduced vitamin K. Non-carboxylation of glutamic acid residues at the amino-terminal ends of factors II, VII, IX and X prevents them from binding to calcium and hence to phospholipids on blood platelets and endothelial cells at the site of injury. The defect in calcium binding results in the inability of these factors to participate in the clotting cascade [7]. Because they do not have any effect on the fully carboxylated clotting factors already in circulation, they do not produce

an immediate anticoagulant effect. The rate of clearance of the previously fully carboxylated factors and the half-life of the coumarin anticoagulant determine the time of onset of the anticoagulant effect [8].

Chemistry and pharmacokinetics of coumarin anticoagulants

The essential chemical characteristics of the coumarin derivatives for anticoagulant activity are an intact 4-hydroxycoumarin residue with a carbon substituent at the 3 position [9]. The most commonly used coumarins are acenocoumarol, phenprocoumon and warfarin. In North America, in the UK and in Scandinavia, warfarin is most commonly prescribed, whereas phenprocoumon and acenocoumarol are the first-line coumarin anticoagulants in other European countries. These compounds have an asymmetrical carbon atom in the substituent at the 3 position, and the available preparations of the drugs are mixtures of the two optical isomers [9]. In phenprocoumon and warfarin, the *S*-(–)-enantiomer has been reported to be more potent with respect to anticoagulant activity, while for acenocoumarol the *R*-(+)-enantiomer seems to be several times more potent [10, 11].

Although the mechanism of action of these 3 coumarin anticoagulants is the same, their pharmacokinetic properties differ to some extent. They are all rapidly, and almost completely absorbed from the upper gastrointestinal tract with little interindividual variation [8]. All coumarin anticoagulants are highly protein bound and have low volumes of distribution. All three drugs are metabolized in the liver by cytochrome P450 enzymes, most importantly CYP2C9, to mostly inactive metabolites, which are excreted in urine and faeces [12-14]. The relative contribution of CYP2C9 to the metabolism differs between the three coumarins, as a consequence of the difference in structure. This enzyme plays a more important role in the oxidation of acenocoumarol and warfarin, than in the oxidation of phenprocoumon. Phenprocoumon is also, for approximately one-third, eliminated as free or conjugated parent-drug [8, 12, 15-17]. The hepatic biotransformation of warfarin appears to be stereoselective. CYP2C9 is the main enzyme that metabolizes the more potent *S*-(–)-enantiomer, whereas the *R*-(+)-enantiomer is preferentially metabolized by other CYP enzymes [14, 18]. For acenocoumarol, CYP2C9 has been identified as the enzyme metabolizing both enantiomers [15]. The elimination half-lives of the coumarin anticoagulants vary widely and thereby the duration of effect. Acenocoumarol is a short-acting anticoagulant with a half-life of 8 to 12 hours. Phenprocoumon has a half-life of 65 to 170 hours and is a long-acting coumarin. Warfarin has an intermediate duration of effect with a half-life of 10 to 45 hours [8, 9]. A number of studies have suggested that longer-acting coumarins give a more stable anticoagulation than short-acting coumarins, which is thought to be associated with greater fluctuations in the plasma levels of factor VII with the shorter-acting drugs [19-21].

Monitoring coumarin anticoagulant therapy

Because underanticoagulation is associated with an exponentially increasing risk of recurrent thrombosis, and overanticoagulation places patients at a heightened risk of bleeding [5, 22-24], anticoagulant therapy needs to be monitored. The benefit-risk ratio of treatment with coumarin anticoagulants is determined to a great extent by the difficulties inherent in the process of achieving and maintaining the therapeutic range of anticoagulation. These stem largely from the high degree of interindividual variability in the dose-response relationship and from the variation within individuals in this relationship over time.

The prothrombin time (PT) test is the most common method for monitoring coumarin anticoagulant therapy [25]. This test responds to reduction of three of the four vitamin K-dependent coagulation factors (II, VII, and X). During the first few days of coumarin anticoagulant therapy, the prolongation of the PT reflects mainly a reduction of factor VII, while subsequently it also reflects a reduction of factors X and II. The PT assay is performed by adding calcium and thromboplastin to citrated plasma. Because commercial thromboplastins have different potencies, the PT can vary widely. In an effort to standardize oral anticoagulation, the international normalised ratio (INR) method has been adopted by most laboratories and clinicians [26]. With this method, the ratio of the patient's PT is compared to the mean PT for a group of normal individuals. The ratio is adjusted for the sensitivity of the laboratory's thromboplastin determined by the International Sensitivity Index (ISI). Thus, INR = (PT patient/PT normal)^{ISI}. Use of the INR permits physicians to obtain the appropriate level of anticoagulation independent of laboratory reagents and to follow published recommendations for intensity of anticoagulation. The optimal target range of coumarin anticoagulant therapy, as recommended by the Federation of Dutch Thrombosis Centers, lies between an INR of 2.5 and 3.5, or between 3.0 and 4.0 [23, 27], depending on the indication for treatment. The necessary duration of treatment ranges from four weeks to lifelong.

Adequate and safe anticoagulation requires experience and a specialized organization [28]. In the Netherlands, the management of individual coumarin anticoagulant therapy is performed at one of 63 regional anticoagulation clinics located throughout the country [29]. Although each anticoagulation centre operates independently, many use one of several available computerised systems to assist with dosing schemes for coumarins. These systems evaluate INR results and in about one-half of cases, make a dosage recommendation that can be modified by the physician [23]. In the other half of cases, consisting mainly of patients who are unstable or have had complications or for whom the prescription of concomitant drugs has changed, the physician adjusts the dosage according to a standard operating procedure without a recommendation from the system. INR measurements and consequent adjustments of the dosing schedules usually occur at intervals of 1 to 6 weeks, dependent on the stability of the anticoagulant level.

In the Netherlands, two coumarin anticoagulants are licensed: acenocoumarol (Sintrom Mitis®) and phenprocoumon (Marcoumar®). The choice of anticoagulant drug is essentially

made by the referring physician and is mainly based on his or her previous experience with these coumarins. Acenocoumarol is prescribed in about 75% of cases, whereas only 25% of the patients are treated with phenprocoumon.

Genetic factors

The wide interindividual variation in coumarin anticoagulant dose requirement is partly genetically determined. Several cytochrome P450 enzymes contribute to oxidative metabolism of these anticoagulants. The most important of these is CYP2C9. The human gene coding for the CYP2C9 protein has been mapped to chromosome 10q24.2 and is greater than 55 kb in length [30, 31]. Over the past several years, multiple single-base pair substitution polymorphisms have been identified. In addition to the wild type allele (CYP2C9*1), at least eleven other CYP2C9 alleles are now known to occur (http://www.imm.ki.se/CYPalleles/). These alleles encode CYP2C9 enzymes with potentially different catalytic activity and specificity [32]. The two most common variant alleles are CYP2C9*2 and CYP2C9*3. Population distribution data suggest that these variant alleles are present in approximately 35% of Caucasian individuals, but are significantly less prevalent in African-American and Asian populations. In several clinical studies, possession of the CYP2C9*2 or CYP2C9*3 variant alleles has been associated with a significant decrease in mean warfarin dose requirement and an increased risk of adverse events, such as bleeding [33-38]. Much less is known about the impact of CYP2C9 polymorphisms on acenocoumarol and phenprocoumon pharmacokinetics and clinical effects. Furthermore, the wide interindividual variation in warfarin dose requirement within the various CYP2C9 genotype groups may suggest that there are also other genetic factors involved.

Environmental factors

In addition to known and unknown genetic factors, demographic factors, various disease states, drugs, and dietary factors can interfere with the response to coumarin anticoagulants. For example, the dosage of warfarin decreases by approximately 10% for each decade of life [39-41]. Hepatic dysfunction potentiates the response to coumarin anticoagulants through impaired synthesis of coagulation factors. Hypermetabolic states produced by fever or hyperthyroidism increase coumarin responsiveness, probably by increasing the catabolism of vitamin K-dependent coagulation factors [42, 43]. In patients with heart failure the increased coumarin responsiveness is reported to be associated with hepatic congestion and redistribution of body water [44-48].

Concomitantly administered drugs can influence the pharmacokinetics of coumarin anticoagulants by altering its metabolic clearance or its rate of absorption from the intestine. Drugs can also influence the pharmacodynamics of coumarin anticoagulants by inhibiting the synthesis of vitamin K-dependent coagulation factors, increasing their metabolic clearance, or interfering with other pathways of hemostasis [5, 49-52]. Lists of drugs that have the potential to interact with coumarin anticoagulants and that have been reported to alter the prothrombin-

time response have been published [49-52], but because of the descriptive nature of most of such reports, no cause-effect relation can usually be inferred from these lists. Only a small number of drug interactions that affect the pharmacokinetics and pharmacodynamics of coumarin anticoagulants have been well documented [53].

Subjects receiving long-term anticoagulant therapy are sensitive to fluctuating levels of dietary vitamin K [54, 55], which is provided predominantly by phylloquinone in plant material [55]. To some extent, vitamin K synthesized by intestinal bacteria may also be available to the human host, but this source is reported to be much less important [51]. There is only limited information in the literature however, on the association between dietary intake of vitamin K and overanticoagulation.

Finally, variability in anticoagulant response also occurs as a result of patient non-compliance, the physician's experience in handling coumarin anticoagulants and miscommunication between patient and physician [5, 56]. However, these topics will not be discussed in this thesis.

Aim and outline of this thesis

This introductory chapter has illustrated that various factors, such as genetic factors, drugs, diet and intercurrent diseases may affect anticoagulation levels. Most of the clinical and pharmacological data related to coumarin anticoagulants have so far been obtained from studying warfarin. Because of the different pharmacokinetic properties of each individual drug, the results of these studies can probably not be directly extrapolated to the other coumarin anticoagulants. Therefore, the aim of this thesis was to study the various genetic and environmental factors affecting the anticoagulation levels of acenocoumarol or phenprocoumon among outpatients of an anticoagulation clinic. In chapter 2 genetic factors influencing the coumarin anticoagulant level are examined. Chapter 2.1 describes how the coumarin anticoagulant dose is affected by polymorphisms of the cytochrome P450 enzyme 2C9 allele. In chapter 2.2 we describe the influence of these polymorphisms on the occurrence of bleeding complications. CYP2C9 variant alleles seem to have several biological consequences. For instance, it has been associated with an increased risk of myocardial infarction in women [57]. Therefore, in chapter 2.3 the risk of myocardial infarction was studied in patients with a CYP2C9 variant allele compared to patients with the wild type genotype. Chapter 2.4 concerns the influence of apolipoprotein E genotype on the coumarin anticoagulant level. In chapter 3, several drugs are identified with a high risk of overanticoagulation during coumarin use. Chapters 3.1 and 3.2 focus on antibacterial drugs and antifungal agents, chapters 3.3 on laxatives, and 3.4 on nonsteroidal anti-inflammatory drugs as risk factors for overanticoagulation. The study described in chapter 4 aims at investigating the influence of heart failure on the anticoagulant level. The role of dietary intake of vitamin K as a risk factor for overanticoagulation is evaluated in chapter 5. Finally, in chapter 6 the strengths and limitations of this thesis are discussed,

together with the implications for coumarin anticoagulant therapy, and suggestions for future research are given.

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Chapter 2

Genetic factors affecting the coumarin anticoagulant level

Chapter 2.1

The risk of overanticoagulation in patients with cytochrome P450 CYP2C9*2 or CYP2C9*3 alleles on acenocoumarol or phenprocoumon

ABSTRACT

Cytochrome P4502C9 (CYP2C9) is the main enzyme implicated in coumarin anticoagulant metabolism. The variant alleles CYP2C9*2 and CYP2C9*3 are associated with an increased response to warfarin. However, an effect on acenocoumarol dose requirements appears to be absent for the CYP2C9*2 allele and the consequences for the metabolism of phenprocoumon have not yet been established. We investigated CYP2C9 polymorphisms in relation to the international normalised ratio (INR) during the first six weeks of treatment and its effect on the maintenance dose in a cohort of 1124 patients from the Rotterdam Study who were treated with acenocoumarol or phenprocoumon. There was a statistically significant difference in first INR between patients with variant genotypes and those with the wild type. Almost all acenocoumarol-treated patients with a variant genotype had a significantly higher mean INR and had a higher risk of an INR $\geq$ 6.0 during the first six weeks of treatment. A clear genotype-dose relationship was found for acenocoumarol-treated patients. For patients on phenprocoumon, no significant differences were found between variant genotypes and the wild type genotype. Individuals with one or more CYP2C9*2 or CYP2C9*3 allele(s) require a significantly lower dose of acenocoumarol compared to wild type patients. Phenprocoumon appears to be a clinically useful alternative in patients carrying the CYP2C9*2 and *3 alleles.

INTRODUCTION

Coumarin anticoagulants are extensively used for the treatment and long-term prevention of thromboembolic diseases [1, 2]. The main drawback is an enhanced risk of hemorrhage [3], which is strongly associated with the intensity of anticoagulation and sharply increases when the international normalised ratio (INR) is $\geq$ 6.0 [4, 5]. Anticoagulant therapy is considered effective and safe if the INR is kept within the therapeutic zone for as long as possible [6, 7]. It is increasingly appreciated that the response to coumarin anticoagulants is largely genetically determined [8]. Cytochrome P450 2C9 (CYP2C9) plays an important role in the metabolism of the coumarin anticoagulants warfarin, phenprocoumon, and acenocoumarol [9, 10]. Warfarin is the main coumarin anticoagulant used in the UK and USA, while acenocoumarol and phenprocoumon are preferentially used in continental Europe [11]. Allelic variants of CYP2C9, CYP2C9*2 ($Arg^{144}Cys$) and CYP2C9*3 ($Ile^{359}Leu$), code for enzymes with approximately 12% and 5% of the enzymatic activity of the wild type genotype CYP2C9*1 (Arg^{144}/Ile^{359}) respectively [12-14]. Both variant alleles have been associated with decreased warfarin dose requirements, more time to achieve stable dosing, a higher risk of bleeding during the initiation phase, and a significantly higher bleeding rate [15-23]. Mannucci [24] has suggested acenocoumarol as an alternative to warfarin in patients carrying the variant alleles CYP2C9*2 and CYP2C9*3. Recently, however, the CYP2C9*3 allele was also associated with low dose requirement for acenocoumarol [25-27]. The effect of the CYP2C9*2 allele on acenocoumarol sensitivity seems to be absent or not clinically relevant [25-27]. To our knowledge, the influence of CYP2C9 polymorphisms on dose requirements in patients on phenprocoumon therapy has not yet been reported. Carriers of the allelic variants will also be at enhanced risk of interactions with drugs that are inhibitors or substrates of CYP2C9 [11]. Most studies on the association between CYP2C9 polymorphisms and dose requirements of coumarin anticoagulants either excluded patients who were taking drugs that may interfere with coumarin metabolism [23, 27] or found no interaction with other CYP2C9 substrates or inhibitors [22, 26]. Furthermore, there is only limited information in the literature on the genotype-dose relationship because many studies did not distinguish between heterozygous and homozygous subjects for the allelic variants.

The aim of the present cohort study was to investigate the effect of the CYP2C9*2 and CYP2C9*3 alleles on the stability of the anticoagulant level during the first six weeks of treatment, and on the maintenance dose of acenocoumarol or phenprocoumon in patients with and without use of CYP2C9 substrates or inhibitors.

METHODS

Setting

Data were obtained from the Rotterdam Study and from the regional outpatient anticoagulation clinic. The Rotterdam Study is a prospective population-based cohort study of neurologic, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, aged 55 years or over were invited in 1990-1993 to participate in the study. The rationale, ethical approval and design of this study have been described elsewhere [28]. The cohort encompasses 7983 individuals who were all interviewed and investigated at baseline. The anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. Almost all patients start with a standard dosing scheme of acenocoumarol (8-4-4 mg during day 1 up to day 3) or phenprocoumon (9-6-3 mg during day 1 up to day 3). Prothrombin times are monitored every one to six weeks by reference to the INR, dependent on the stability of the anticoagulant level. Doses are adjusted on the basis of the INR of the patient by computerised dose calculations. All data on dosing, laboratory, and clinical data as of 1984 are fully computerised. For this study, data were used from January 1, 1985 through December 31, 1998. For data on comedication, we used data from seven regional pharmacies where more than 99% of participants of the Rotterdam Study fill their prescriptions. Complete data on drug use from these pharmacies were available as of January 1, 1991. For those patients for whom we compared maintenance doses of coumarin anticoagulants during treatment episodes with and without CYP2C9 comedication, we therefore used only the data from the anticoagulation clinic after January 1, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC) code [29], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs.

Cohort definition

The study cohort consisted of all 1312 patients in the Rotterdam Study, who started treatment with acenocoumarol or phenprocoumon in the study period between January 1, 1985 and December 31, 1998 and for whom there were INR data during their treatment. If a patient had multiple treatment episodes during the study period, all episodes were considered. We defined the follow-up period as the sum of all treatment episodes per patient. The cohort was followed until the last INR assessment, because of the end of their treatment, death or end of the study period, whichever came first.

Outcomes

The average first and second INR on the standard start dosing scheme of acenocoumarol or phenprocoumon were calculated per CYP2C9 genotype. For each individual patient, the mean number of INR assessments, the mean INR and the occurrence of an INR $\geq$ 6.0 during the first

six weeks after start of the coumarin anticoagulant were assessed. The mean number of INR assessments was used as a measure of instability of the anticoagulant level. Patients who had a follow-up period of more than six weeks were included in the analyses on maintenance dose. The maintenance dose was defined as the mean dose (in mg per week) calculated over all treatment episodes from day 43 up to the end of each treatment episode. The mean INR was calculated over the same period. For patients who had treatment episodes with and without use of other CYP2C9 comedication, mean doses and mean INR were separately calculated during both episodes. The following substrates and inhibitors of the CYPC9 enzyme were considered as CYP2C9 comedication: amiodarone, carbamazepine, chloramphenicol, cimetidine, diclofenac, fluconazole, fluvastatine, losartan, miconazole, phenylbutazone, phenytoin, sulphadiazine, sulphamethizole, sulphamethoxazole, sulphinpyrazone, tolbutamide, trimethoprim, and zafirlukast [30].

Cofactors

The following patient characteristics were considered as potential determinants for affecting the coumarin maintenance dose: age, sex and body mass index. In addition, we considered the target INR (low, medium and high) as well as the mean INR level after the first six weeks of treatment.

Genotyping

Genotyping for the CYP2C9*2 and CYP2C9*3 allele variants was performed by using polymerase chain reaction followed by restriction enzyme digestion analysis (PCR-RFLP), as previously described by Aynacioglu et al [31]. Approximately 5 ng of genomic DNA was amplified in 35 cycles of PCR: 1 min 94°C, 1 min 60°C (CYP2C9*2) or 1 min 62°C (CYP2C9*3) and 1 min 72°C, in a total volume of 10 µl, using primers P141 (5'-CACTGGCTGAAAGAGCTAACAGAG-3') and P142 (5'-GTGATATGGAGTAGGGTCACCCAC-3') for CYP2C9*2, or P143 (5'-AGGAAGAGATTGAACGTGTGA-3') and P144 (5'-GGCAGGCTGGTGGGGAGAAGG**C**CAA-3') for CYP2C9*3 (the bold nucleotide represents a mismatch to the genomic sequence). The PCR product was digested with Sau96 (CYP2C9*2) or StyI (CYP2C9*3), and analysed on a 3% TBE/agarose gel with ethidium bromide staining. Assays were validated by direct sequencing (Big Dye terminator chemistry, Applied Biosystems, Foster City, CA, USA) on an ABI 310 genetic analyser (Applied Biosystems). All CYP2C9*2 and CYP2C9*3 heterozygote and homozygote variants detected were reanalysed. Patients in whom neither CYP2C9*2 nor CYP2C9*3 alleles were identified were regarded as wild type.

Statistical analysis

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by using a χ^2-test. Independent-samples t-tests were used to compare the mean number of INR assessments and the mean INR during the first six weeks of treatment and during the rest of the

follow-up between carriers of variant alleles and the wild type genotype. Relative risks (RR) with 95% confidence intervals (CI) were calculated to compare the proportion of patients with an INR $\geq$ 6.0 between variant genotypes and the wild type genotype. For patients who had episodes of coumarin anticoagulant therapy with and without CYP2C9 comedication, paired-sampled t-tests were used to compare the difference in mean maintenance dose and mean INR between these episodes. A multivariate linear regression model was used to assess the effect of allelic variants on the coumarin maintenance dose, while controlling for differences in cofactors. This was only carried out for the treatment episodes without use of CYP2C9 comedication. Cofactors were included in the model if the point estimate changed by more than 5% upon inclusion of the cofactor in the model. All statistical analyses were performed with SPSS software (version 10.0; SPSS, Chicago, Illinois, USA). To evaluate a genotype-dose relationship, a trend test was performed using a linear regression model with the mean maintenance dose as outcome variable and the genotypes as an ordinal set with 5 values.

RESULTS

Of the 1312 patients in the cohort, 29 were excluded because they used other coumarin anticoagulants than acenocoumarol or phenprocoumon, and 159 patients were excluded because of difficulties in genotyping (due to the suboptimal quality of the long-term storage of DNA of some samples). Consequently, there were 1124 patients available for analysis (Table 1). The mean age of these patients was approximately 72 years, and 47.2% of the patients were men. All patients were of Caucasian origin. There were 970 acenocoumarol-treated patients (86.3%) and 204 patients (18.1%) who used phenprocoumon. Fifty of these used both coumarin anticoagulants during the study period. The main indications for anticoagulation were: treatment of deep venous thrombosis, pulmonary embolism and short-term prophylactic treatment (low target INR level (2.5-3.5); 39.0% of patients), atrial fibrillation, myocardial infarction, coronary bypass, vascular surgery, stroke and transient ischaemic attacks (medium target INR level (3.0-4.0); 58.9%), and prosthetic heart valve (high target INR level (3.5-4.5); 2.1%). The range of the weekly maintenance dose was 1-56 mg for acenocoumarol and 0.8-84 mg for phenprocoumon. The mean follow-up time was 598 days (1.8 years). Patients had a median of 20 INR assessments during a median follow-up time of 279 days (0.8 years). There were 771 patients (68.6%) with the wild type genotype, and 353 (31.4%) with a variant genotype. The frequencies of the CYP2C9*2 and CYP2C9*3 alleles were 13.5% and 4.0%, respectively. The cohort was in Hardy-Weinberg equilibrium when variant alleles were taken together ($p = 0.14$). However, if the data were calculated only for the CYP2C9*3 allele, a lack of equilibrium were observed since no individuals homozygous for this allele were identified.

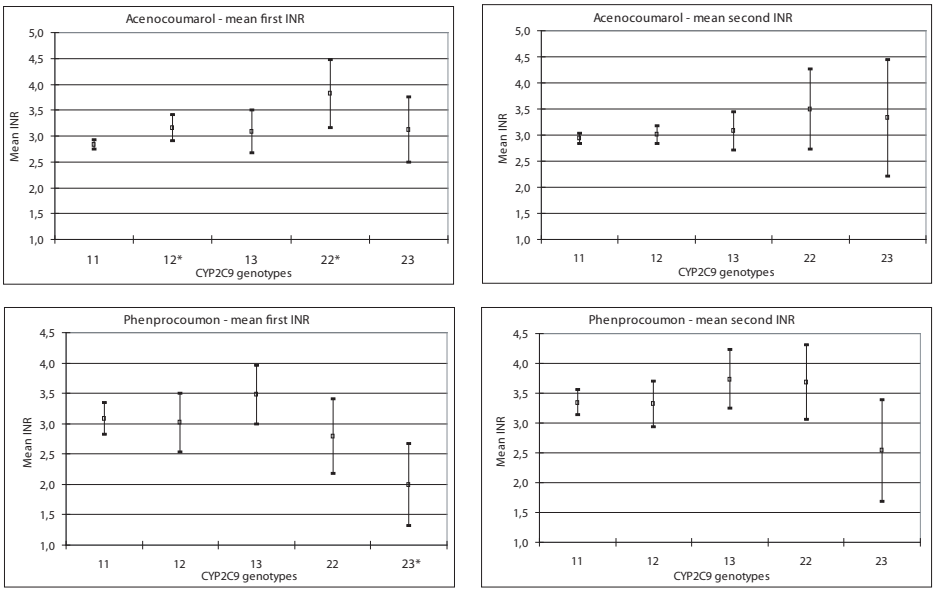
The mean first INR after a standard starting dose of acenocoumarol was higher in all variant genotypes than in the wild type genotype (95%CI: 2.7-2.9), but the differences compared to

Table 1. Characteristics of the study population

Variable	Number of patients (n = 1124)
Gender	
Male	530 (47.2%)
Female	594 (52.8%)
Age, average (SD)	71.8 (8.1) years
Caucasian origin	1124 (100%)
Type of coumarin*	
Acenocoumarol	970 (86.3%)
Phenprocoumon	204 (18.1%)
Target INR level	
Low (2.5-3.5 INR)	438 (39.0%)
Medium (3.0-4.0 INR)	662 (58.9%)
High (3.5-4.5 INR)	24 (2.1%)
Weekly maintenance dose range	
Acenocoumarol	1 - 56 mg
Phenprocoumon	0.8 - 84 mg
Follow-up time	
Mean	598 days
Median	279 days
Genotype§	
CYP2C9*1/*1	771 (68.6%)
CYP2C9*1/*2	239 (21.3%)
CYP2C9*1/*3	73 (6.5%)
CYP2C9*2/*2	23 (2.0%)
CYP2C9*2/*3	18 (1.6%)
CYP2C9*3/*3	-

* Because 50 patients use both coumarins at any time during the study period, the total percentage is more than 100.
§ Hardy-Weinberg Equilibrium: $\chi^{2=}5.435$ ($p = 0.14$).

the wild type were only statistically significant for the CYP2C9*1/*2 (95%CI: 2.9-3.4) and the CYP2C9*2/*2 (95%CI: 3.2-4.5) genotypes (Figure 1). These differences could not be explained by the fact that patients with a variant genotype were targeted at a higher INR level ($p = 0.915$ for difference in target level between genotypes). For the second mean INR on acenocoumarol there were no significant differences anymore between the variant and the wild type genotypes. For phenprocoumon-treated patients the first INR tended to be lower in the variant than in the wild type genotype (95%CI: 2.8-3.4), but the difference only reached statistical significance for the CYP2C9*2/*3 genotype (95%CI: 1.3-2.7). Also for phenprocoumon, there were no statistically significant differences for the second INR. The mean number of INR assessments, the mean INR and the relative risk of overanticoagulation during the first 6 weeks after start of the coumarin anticoagulant are shown in Table 2 for all CYP2C9 genotypes. For acenocoumarol- and phenprocoumon-treated patients, there were no statistically significant differences in the mean number of INR assessments between variant genotypes and the wild



□, mean INR; bars represent the 95% confidence intervals

* genotypes with a statistically significant different mean INR

Figure 1. Mean first and second INR for patients with standard start doses of acenocoumarol and phenprocoumon

Table 2. The mean number of INR assessments, the mean INR and the relative risk of overanticoagulation in relation to CYP2C9 genotype during the first 6 weeks after starting with acenocoumarol or phenprocoumon

Genotype	N pat	Mean n INR assessments (SEM*)	P†	Mean INR (SEM*)	P‡	N pat (%) with an INR ≥ 6.0	RR (95%CI)§
Acenocoumarol	970						
*1/*1	669	6.2 (0.12)	-	2.9 (0.03)	-	58 (8.7)	-
*1/*2	208	6.1 (0.21)	0.68	3.1 (0.05)	0.04	24 (11.5)	1.4 (0.8-2.3)
*1/*3	57	5.9 (0.36)	0.49	3.1 (0.09)	0.15	8 (14.0)	1.7 (0.8-3.8)
*2/*2	20	7.1 (0.93)	0.34	3.5 (0.19)	0.001	5 (25.0)	3.5 (1.2-10)
*2/*3	16	7.0 (1.09)	0.47	3.2 (0.12)	0.07	3 (18.8)	2.4 (0.7-8.8)
Phenprocoumon	204						
*1/*1	134	5.1 (0.21)	-	3.5 (0.07)	-	22 (16.4)	-
*1/*2	40	5.0 (0.36)	0.86	3.5 (0.11)	0.85	7 (17.5)	1.1 (0.4-2.8)
*1/*3	19	5.6 (0.84)	0.38	3.7 (0.17)	0.22	3 (15.8)	1.0 (0.3-3.6)
*2/*2	7	4.1 (0.77)	0.34	3.7 (0.42)	0.49	1 (14.3)	0.8 (0.1-7.4)
*2/*3	4	6.3 (1.65)	0.35	2.8 (0.14)	0.10	-	-

* Standard error of the mean.

† P-value for the difference in mean number of INR assessments between carriers of a variant allele and the wild type genotype.

‡ P-value for the difference in mean INR between carriers of a variant allele and the wild type genotype.

§ Relative risk (RR) of experiencing an INR ≥ 6.0 for carriers of a variant allele compared to the wild type genotype.

type genotype. The mean INR in patients on acenocoumarol was significantly higher in the CYP2C9*1/*2, CYP2C9*2/*2 and CYP2C9*2/*3 genotypes than in the wild type genotype. For phenprocoumon-treated patients, there were no statistically significant differences in mean INR between variant and wild type genotypes. There were 98 patients on acenocoumarol with an INR ≥ 6.0 during the first 6 weeks of treatment of whom 11 (11.2%) experienced a bleeding, and 33 on phenprocoumon with an INR ≥ 6.0 of whom 8 (24%) developed a bleeding. These numbers were too low for demonstrating a statistically significant difference between variant and wild type alleles. Although the percentage of acenocoumarol-treated patients with an INR ≥ 6.0 tended to increase (trend test p = 0.006) when comparing CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2 and CYP2C9*2/*3 genotypes with the CYP2C9*1/*1 wild type, the relative risk was only statistically significantly increased for the CYP2C9*2/*2 genotype (RR 3.5; 95%CI: 1.2-10.0). Patients with a variant genotype on phenprocoumon had no higher risk of overanticoagulation during the first 6 weeks of anticoagulant therapy.

Of the 970 patients who started with acenocoumarol during the study period, 835 had a follow-up period longer than 6 weeks and, for 754 of them, we had data available on acenocoumarol doses without use of CYP2C9 comedication. Of the 204 patients who started with phenprocoumon, 184 patients used the drug long enough to calculate maintenance doses and, for 173 of them, we had data available on the phenprocoumon dose without use of CYP2C9 comedication. The 143 short-term users of acenocoumarol (p = 0.3) and 31 short-term users of phenprocoumon (p = 0.8) had a similar proportion of variant alleles as long-term users. Table 3 summarizes the mean maintenance doses of acenocoumarol and phenprocoumon and the mean INR in relation to the CYP2C9 genotype during the treatment episodes without use

Table 3. Mean maintenance dose and mean INR in relation to CYP2C9 genotype during treatment episodes without CYP2C9 comedication

Genotype	N pat	Mean dose (mg/week) (SEM*)	Difference with *1/*1 (95%CI)†	Mean INR (SEM*)	Difference with *1/*1 (95%CI)‡
Acenocoumarol	754				
*1/*1	526	17.9 (0.31)	-	3.2 (0.03)	-
*1/*2	159	15.5 (0.51)	-2.3 [-3.5-(-1.2)]	3.3 (0.09)	0.1 (-0.02-0.2)
*1/*3	46	13.9 (0.91)	-3.5 [-5.4-(-1.5)]	3.2 (0.08)	0.01 (-0.2-0.2)
*2/*2	15	13.1 (1.56)	-5.0 [-8.4-(-1.7)]	3.4 (0.12)	0.3 (-0.02-0.6)
*2/*3	8	11.8 (1.76)	-7.2 [-11.7-(-2.6)]	3.1 (0.16)	-0.03 (-0.4-0.4)
Phenprocoumon	173				
*1/*1	114	15.6 (0.66)	-	3.5 (0.08)	-
*1/*2	35	14.0 (1.00)	-1.6 (-4.0-0.9)	3.5 (0.07)	-0.05 (-0.3-0.2)
*1/*3	16	12.9 (1.65)	-2.7 (-6.1-0.7)	3.2 (0.17)	-0.3 (-0.8-0.1)
*2/*2	5	10.0 (1.79)	-5.2 (-11.0-0.7)	3.3 (0.21)	-0.3 (-1.1-0.5)
*2/*3	3	16.7 (5.46)	-1.4 (-6.2-9.1)	3.6 (0.29)	-0.02 (-1.0-1.0)

* Standard error of the mean.
† Difference in mean dose compared to the wild type genotype with a 95% confidence interval, adjusted for differences in age and mean INR.
‡ Difference in mean INR compared to the wild type genotype with a 95% confidence interval.

of CYP2C9 comedication. The difference in mean maintenance dose, adjusted for differences in cofactors, and the difference in mean INR compared to the wild type genotype are also shown. For acenocoumarol, a genotype-dose relationship is suggested when comparing the CYP2C9*1/*1, CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2 and CYP2C9*2/*3 genotypes, with average maintenance doses of 17.9, 15.5, 13.9, 13.1 and 11.8 mg per week, respectively (trend test p<0.001). Also patients with only one CYP2C9*2 allele required a statistically significant lower dose of acenocoumarol than patients with the wild type genotype. The maintenance dose in patients with variant alleles on phenprocoumon was somewhat lower than in patients with the wild type genotype but this difference was not statistically significant, nor was there any decreasing trend (p = 0.176). Age was an important factor in modulating the response to coumarin anticoagulants. For every year of age, there was a statistically significant decrease in acenocoumarol dose of 0.30 (95%CI: 0.24-0.36) mg per week. For phenprocoumon, the dose decrease was 0.19 (95%CI: 0.07-0.32) mg per week for every year of age. Neither sex, body mass index, nor the target INR level affected the mean maintenance dose of acenocoumarol or phenprocoumon.

Of the 283 patients who used acenocoumarol with and without CYP2C9 comedication, the maintenance dose was 0.6 mg per week lower during the treatment episodes with CYP2C9 comedication (p < 0.001), while the mean INR was 0.1 unit higher during these episodes (p = 0.008). For the 74 patients using phenprocoumon with and without CYP2C9 comedication, there was no statistically significant difference in mean maintenance dose (p = 0.596), nor in mean INR (p = 0.423), between the treatment episodes with and without CYP2C9 comedication. Numbers were too low to stratify these data on CYP2C9 genotype.

DISCUSSION

The main finding in this population-based cohort study is that individuals heterozygous for either CYP2C9*2 or CYP2C9*3, as well as those with two variant CYP2C9 alleles, required a significantly lower dose of acenocoumarol to reach the same level of anticoagulation compared to patients with the wild type genotype. We found a clear genotype-dose relationship for acenocoumarol-treated patients when comparing the CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2 and CYP2C9*2/*3 genotypes with the wild type. The acenocoumarol maintenance doses for the CYP2C9 genotypes are in line with those recently published by Tassies et al. [27], except for the CYP2C9*2/*2 variant. They found similar maintenance doses for the CYP2C9*2/*2 and the wild type genotype (17.0 and 17.1 mg per week, respectively), while the maintenance dose for patients with the CYP2C9*1/*2 genotype was 14.4 mg per week. However, these doses were not adjusted for confounding factors. For phenprocoumon we did not find the same results as for acenocoumarol. This could partly be explained by the fact that the cytochrome P450 2C9 enzyme plays a more important role in the oxidation of acenocoumarol and warfarin, than in

the oxidation of phenprocoumon [9, 10]. On the other hand, numbers were small for some genotypes in phenprocoumon-treated patients, and this could have resulted in less precise estimates.

Due to the fact that CYP2C9 plays a more substantial role in the metabolism of acenocoumarol, patients treated with this coumarin anticoagulant may be more sensitive to interference by the use of other CYP2C9 substrates or inhibitors. As the reduction in maintenance dose in patients who had treatment episodes with, as well as without, CYP2C9 comedication was small but highly significant in acenocoumarol-treated patients, these results are in line with ours. There was no difference between these treatment episodes for patients on phenprocoumon.

During the initiation of the anticoagulant therapy several differences were found between variant genotypes and wild type patients. For acenocoumarol-treated patients, the first INR and the mean INR during the first 6 weeks of treatment tended to be higher in the variant genotypes than in the wild type patients, although the differences did not reach statistical significance in all variant genotypes. Patients with a variant genotype also appear to be at a higher risk of overanticoagulation than wild type patients. The reason why the differences in the first INR between the wild type and the variant genotypes were not present anymore during the second INR measurement is that, in anticoagulation clinics, doses are adjusted according to the INR-value obtained during the first measurement. For patients on phenprocoumon, there were no differences in these parameters between variant genotypes and the wild type patients. Why the first INR in the variant genotypes on phenprocoumon tended to be lower than in the wild type patients, could possibly be explained by the fact that some of these patients switched from acenocoumarol to phenprocoumon because anticoagulant levels remained highly unstable on acenocoumarol, and they therefore started with a lower dose of phenprocoumon. Unfortunately, we had no data on individual starting doses to evaluate this. We defined the initiation phase of the anticoagulant therapy as the first 6 weeks of treatment, whereas this is sometimes defined as the first month of treatment. However, when we shortened our initiation phase to the first month of treatment, the results were similar.

The allele frequency for CYP2C9*2 found in our population falls within the wide range of reported allele frequencies among various Caucasian populations (8-19%) and is very close to those found in other Dutch, German and British populations [32]. Caucasians also exhibit a significant heterogeneity in CYP2C9*3 allele frequency (3.3-16.2%). Although the allele frequency for CYP2C9*3 in our population falls within this range, it is lower than those reported in other northern European countries [32]. The study population is at Hardy-Weinberg equilibrium for overall variant alleles. The expected number of patients homozygous for CYP2C9*3 is less than 2, and it is not surprising that no individuals were identified in our study population who were homozygous for CYP2C9*3. For phenotype prediction, variant alleles CYP2C9*2 and CYP2C9*3 can be taken together because there is no phenotype/genotype disagreement in the metabolism of coumarin anticoagulants for these variant alleles [33].

In conclusion, individuals heterozygous for either CYP2C9*2 or CYP2C9*3, as well as those with two variant CYP2C9 alleles, require a significantly lower dose of acenocoumarol than wild type patients to reach the same level of anticoagulation. Patients who use other CYP2C9 substrates or inhibitors concomitantly need a significant lower dose of acenocoumarol. Phenprocoumon appears to be a useful clinical alternative in patients carrying the CYP2C9*2 and *3 alleles.

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Chapter 2.2

The risk of bleeding complications in patients with cytochrome P450 CYP2C9*2 or CYP2C9*3 alleles on acenocoumarol or phenprocoumon

ABSTRACT

Introduction: The principal enzyme involved in coumarin metabolism is CYP2C9. Allelic variants of CYP2C9, CYP2C9*2 and CYP2C9*3, code for enzymes with reduced activity. Despite increasing evidence that patients with these genetic variants require lower maintenance doses of anticoagulant therapy, there is lack of agreement among studies on the risk of bleeding and CYP2C9 polymorphisms.

Objective: To study the effect of the CYP2C9 polymorphisms on bleeding complications during initiation and maintenance phases of coumarin anticoagulant therapy.

Design: Population-based cohort study in a sample of the Rotterdam Study, a study in 7983 subjects.

Subjects: All patients who started treatment with acenocoumarol or phenprocoumon in the study period from January 1, 1991 through December 31, 1998 and for whom INR data were available.

Methods: Patients were followed until a bleeding complication, the end of their treatment, death or end of the study period. Proportional hazards regression analysis was used to estimate the risk of a bleeding complication in relation to CYP2C9 genotype after adjustment for several potentially confounding factors such as age, gender, target INR level, INR, time between INR measurements, and aspirin use. The effect of variant genotype on bleeding risk was separately examined during the initiation phase of 90 days after starting therapy with coumarins.

Results: The 996 patients with analysable data had a mean follow-up time of 481 days (1.3 years); 311 (31.2%) had at least 1 variant CYP2C9 allele and 685 (68.8%) had the wild type genotype. For patients with the wild type genotype, the rate of minor bleeding, major bleeding and fatal bleeding was 15.9, 3.4 and 0.2 per 100 treatment-years, respectively. For patients with a variant genotype, the rate of minor, major and fatal bleeding was 14.6, 5.4 and 0.5 per 100 treatment-years. Patients with a variant genotype on acenocoumarol had a significantly increased risk for a major bleeding event (RR 1.83, 95%CI: 1.01-3.32). During the initiation phase of therapy we found no effect of variant genotype on bleeding risk.

Conclusion: In this study among outpatients of an anticoagulation clinic using acenocoumarol or phenprocoumon, having a variant allele of CYP2C9 was associated with an increased risk of major bleeding events in patients on acenocoumarol, but not in patients on phenprocoumon. Although one might consider the assessment of the CYP2C9 genotype of a patient for dose adjustment before starting treatment with acenocoumarol, a prospective randomised trial should demonstrate whether this reduces the increased risk of major bleeding events.

INTRODUCTION

Coumarin anticoagulants are extensively used for the treatment and long-term prevention of thromboembolic diseases [1, 2]. The main drawback is an enhanced risk of hemorrhage [3], which is strongly associated with the intensity of anticoagulation and sharply increases when the international normalised ratio (INR) is ≥ 6.0 [4, 5].

There is increasing evidence that the response to coumarin anticoagulants is largely genetically determined [6]. Cytochrome P450 2C9 (CYP2C9) is the major enzyme involved in the metabolism of the coumarin anticoagulants warfarin, phenprocoumon, and acenocoumarol [7, 8]. Warfarin is the main coumarin anticoagulant used in the United Kingdom and United States, while acenocoumarol and phenprocoumon are preferentially used in continental Europe [9]. Allelic variants of CYP2C9, CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu), code for enzymes with approximately 12% and 5% of the enzymatic activity of the wild type genotype CYP2C9*1 (Arg144/Ile359), respectively [10-12]. Bleeding complications have been related to CYP2C9 polymorphisms in several studies in patients receiving warfarin, but these findings could not be confirmed by others [13-17]. Tassies et al [18] were the first who reported on the influence of CYP2C9 polymorphisms on bleeding complications in patients on acenocoumarol therapy. They found no association between repeated bleeding episodes and CYP2C9 polymorphisms. Recently, Hummers-Pradier et al [19] reported an increased risk of bleeding in carriers of a CYP2C9*3 allele who were anticoagulated with phenprocoumon. The lack of agreement among studies on the risk of bleeding and CYP2C9 polymorphisms may have several explanations, including differences in selected populations, the presence of additional predisposing bleeding factors, the different coumarin anticoagulants under study, and the relatively small numbers of patients with variant genotypes. It has also been argued that the risk of bleeding may be especially high at the initiation of therapy in carriers of variant alleles, suggesting that once antithrombotic stability is attained, experienced clinicians are most likely to maintain a patient's INR and minimize their bleeding risk regardless of CYP2C9 genotype [15, 16].

The aim of the present study was to investigate the effect of the CYP2C9 polymorphisms on bleeding complications during initiation and maintenance phases of anticoagulant therapy, in a large population-based cohort of acenocoumarol- and phenprocoumon-treated patients.

METHODS

Setting

Data were obtained from the Rotterdam Study, from the regional outpatient anticoagulation clinic and from hospitals in the Rotterdam area. The Rotterdam Study is a prospective population-based cohort study of neurological, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands,

aged 55 years or over were invited in 1990-1993 to participate in the study. The Medical Ethics Committee of the Erasmus MC approved the study, and written informed consent was obtained from all participants. The rationale and design of this study have been described elsewhere [20]. The cohort encompasses 7983 individuals who were all interviewed and investigated at baseline. At the baseline examination of the Rotterdam Study, blood was taken and DNA was isolated. Since the start of the Rotterdam Study, cross-sectional surveys have been carried out periodically. In addition, participants are continuously monitored for major events through automated linkage with the files from the general practitioners of the patients. When an event or death has been reported, additional information is obtained by interviewing the GP and scrutinising information from hospital discharge records in case of admittance or referral. The anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. Prothrombin times are monitored each one to six weeks by reference to the INR, target range, and stability of the anticoagulant level. Dosing of the coumarin is performed by a team of specialized physicians, with the aid of a computerized dosing program. This program evaluates the stability of the INR and, when possible, proposes a dosing schedule (i.e. in nearly 50% of the patients). In the other patients, dosing is done by the physician according to a standard operating procedure. All data on dosing, laboratory-, and clinical data (e.g. bleeding complications, hospital admissions) are fully computerised. Apart from data from the Rotterdam Study and from the anticoagulation clinic, we used hospital discharge diagnoses for case finding, gathered from all hospitals in the Rotterdam area. Hospital records were linked to the Rotterdam Study database. For potential bleeding cases identified in this way, copies of discharge letters were collected.

Cohort definition

The study cohort consisted of all 1135 patients in the Rotterdam Study, who started treatment with acenocoumarol or phenprocoumon in the study period between January 1, 1991 and December 31, 1998 and for whom there were INR data during their treatment. If a patient had multiple treatment episodes during the study period, only the first episode was considered. If a patient switched from one coumarin to the other, only the first coumarin was considered. The cohort was followed until the first occurrence of a bleeding complication, the last INR assessment because of the end of their treatment, death or end of the study period, whichever came first.

Outcomes

Bleeding complications were subdivided into major and minor bleeding. Hemorrhages were classified as major if these led to death, necessitated hospitalisation, blood transfusion or surgery, or if these concerned intracranial-, intra-articular- or intra-muscular bleeding. Minor bleeding included all other bleeding complications, including skin bleeds of more than 10 cm in diameter and nose bleeds lasting for at least 30 minutes.

Genotyping

Genotyping for the CYP2C9*2 and CYP2C9*3 allele variants was performed by using polymerase chain reaction followed by restriction enzyme digestion analysis (PCR-RFLP), as previously described by Aynacioglu et al [21]. Approximately 5 ng of genomic DNA was amplified in 35 cycles of PCR: 1 min 94°C, 1 min 60°C (CYP2C9*2) or 1 min 62°C (CYP2C9*3) and 1 min 72°C, in a total volume of 10 µl, using primers P141 (5'-CACTGGCTGAAAGAGCTAACAGAG-3') and P142 (5'-GTGATATGGAGTAGGGTCACCCAC-3') for CYP2C9*2, or P143 (5'-AGGAAGAGATTGAACGTGTGA-3') and P144 (5'-GGCAGGCTGGTGGGGAGAAGG**C**CAA-3') for CYP2C9*3 (the bold and underlined nucleotide represents a mismatch to the genomic sequence). The PCR product was digested with Sau96 (CYP2C9*2) or StyI (CYP2C9*3), and analyzed on a 3% TBE/agarose gel with ethidium bromide staining. All CYP2C9*2 and CYP2C9*3 heterozygote and homozygote variants detected were reanalyzed. Patients in whom neither CYP2C9*2 nor CYP2C9*3 alleles were identified were regarded as wild type.

Statistical analysis

Genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by using a χ^2-test. Incidence rates of bleeding complications were calculated in the standard way, by dividing the number of events by the number of treatment-years. The association between CYP2C9 genotype and bleeding complications was evaluated using survival analysis techniques. Patients were divided into 2 groups based on genotype: wild type (CYP2C9*1/*1 homozygotes) and variant (1 or more of the mutant alleles CYP2C9*2 or CYP2C9*3). For each analysis, a relative risk (RR) and 95% confidence interval (95%CI) comparing variant and wildtype genotype groups were computed. We used Cox proportional hazards models to adjust for the potential confounding effect of age, gender, target INR level, INR, time between INR measurements, and aspirin use. We examined the effect of variant genotype on bleeding risk during the initiation phase of therapy by censoring the data at 90 days after starting therapy with coumarins. To study whether there is effect modification by the type of coumarin, stratified analyses were performed for subjects on acenocoumarol and phenprocoumon. Moreover, relative risks were separately calculated for carriers of a CYP2C9*2 allele (CYP2C9*1/*2 and CYP2C9*2/*2) and of a CYP2C9*3 allele (CYP2C9*1/3, CYP2C9*2/*3, and CYP2C9*3/*3). All statistical analyses were performed with SPSS software (version 10.0; SPSS, Chicago, IL). P values less than 0.05 were considered statistically significant.

RESULTS

Of the 1135 patients in the cohort, 139 were excluded because of difficulties in genotyping (due to the suboptimal quality of the long-term storage of DNA of some samples). Consequently, there were 996 patients available for analysis (Table 1). The mean age of these patients was

Table 1. Characteristics of the study population

Variable	Number of patients (n = 996)
Age, average (SD)	71.4 (7.9) years
Gender	
Male	473 (47.5%)
Female	523 (52.5%)
Caucasian origin	996 (100%)
Type of coumarin	
Acenocoumarol	841 (84.4%)
Phenprocoumon	155 (15.6%)
Target INR level	
Low (2.5-3.5)	365 (36.6%)
Medium (3.0-4.0)	607 (60.9%)
High (3.5-4.5)	24 (2.4%)
Follow-up time	
Mean	481 days
Median	210 days
Genotype*	
CYP2C9*1/*1	685 (68.8%)
CYP2C9*1/*2	210 (21.1%)
CYP2C9*1/*3	63 (6.3%)
CYP2C9*2/*2	23 (2.3%)
CYP2C9*2/*3	15 (1.5%)
CYP2C9*3/*3	0 (0%)

* Hardy Weinberg Equilibrium; $\chi^2 = 5.400$ ($p = 0.14$).

71 years, and 47.5% of the patients were men. All patients were of Caucasian origin. There were 841 acenocoumarol-treated patients (84.4%) and 155 patients (15.6%) who used phenprocoumon. The main indications for anticoagulation were: treatment of deep venous thrombosis, pulmonary embolism and short-term prophylactic treatment (low target INR level (2.5-3.5); 36.6% of patients), atrial fibrillation, myocardial infarction, coronary bypass, vascular surgery, stroke and transient ischaemic attacks (medium target INR level (3.0-4.0); 60.9%), and prosthetic heart valve (high target INR level (3.5-4.5); 2.4%). The mean follow-up time was 481 days (1.3 years). Patients had a median of 17 INR assessments during a median follow-up time of 210 days (0.6 years). There were 685 patients (68.8%) with the wild type genotype, and 311 (31.2%) with a variant genotype. The frequencies of the CYP2C9*2 and CYP2C9*3 alleles were 13.6% and 3.9%, respectively. Genotype proportions were in Hardy-Weinberg equilibrium ($p = 0.14$).

The total number of bleeding episodes was 255, or 19.4 per 100 treatment-years (Table 2). Major bleeding occurred 4.0 times per 100 treatment-years, and in 4 cases bleeding resulted in death (0.3 per 100 treatment-years). The most frequent major bleeding complications were bleeding from the gastrointestinal tract, intracranial bleeding and gross hematuria. The mean

Table 2. Observed bleeding complications (per 100 treatment-years) and relative risks (RR) for bleeding complications in patients having the CYP2C9 variant genotype

	Total	Variant genotype	Wild type genotype	RR (95%CI)	
Number patients	996	311	685	Unadjusted	Adjusted
Treatment-years	1311.7	390.9	920.7		
All bleeding	255 (19.4)	78 (20.0)	177 (19.2)	1.03 (0.79-1.34)	0.98 (0.75-1.29)
Minor bleeding	203 (15.5)	57 (14.6)	146 (15.9)	0.91 (0.67-1.24)	0.87 (0.64-1.18)
Major bleeding	52 (4.0)	21 (5.4)	31 (3.4)	1.58 (0.91-2.75)	1.57 (0.90-2.75)
Digestive tract	28 (2.1)	12 (3.1)	16 (1.7)	1.74 (0.83-3.69)	1.72 (0.81-3.66)
Intracranial	10 (0.76)	4 (1.0)	6 (0.65)	1.57 (0.44-5.57)	1.97 (0.53-7.29)
Hematuria	3 (0.23)	1 (0.26)	2 (0.22)	1.16 (0.11-12.8)	-
Muscle joint hematoma	2 (0.15)	1 (0.26)	1 (0.11)	2.35 (0.15-37.5)	-
Other	9 (0.69)	3 (0.77)	6 (0.65)	1.16 (0.29-4.64)	1.04 (0.26-4.21)
Fatal bleeding	4 (0.30)	2 (0.51)	2 (0.22)	2.32 (0.33-16.5)	3.37 (0.41-27.8)

INR (± SD) at the time of bleeding was 3.7 (± 1.9) for the minor bleeding complications and 5.4 (± 5.1) for the major bleeding complications. This difference was however not statistically significant (p = 0.25). For patients with the wild type genotype, the rate of minor bleeding (n = 146), major bleeding (n = 31) and fatal bleeding (n = 2) was 15.9, 3.4 and 0.2 per 100 treatment-years, respectively. For patients with a variant allele, the rate of minor bleeding events (n = 57) was 14.6 per 100 treatment-years, the rate of major bleeding events (n = 21) was 5.4 per 100 treatment-years, and the rate of fatal bleeding events (n = 2) was 0.5 per 100 treatment-years. Kaplan-Meier curves for time to a major bleeding event are shown in Figure 1. During the initiation phase of therapy there appeared to be no effect of genotype on the risk of a major bleeding event. The difference between variant genotypes and wild type patients became apparent after 460 days of anticoagulant therapy. For all bleeding and minor bleeding events, we did not find an increased risk of bleeding in relation to variant genotype (Table 2). Patients with a variant genotype had a 57% higher risk of a major bleeding complication compared to patients with the wild type genotype, although this difference was not statistically significant. Relative risks for patients on acenocoumarol were 1.05 (95%CI: 0.78-1.42) for all bleeding, 0.89 (95%CI: 0.63-1.26) for minor bleeding and 1.83 (95%CI: 1.01-3.32) for major bleeding complications. Relative risks for phenprocoumon-treated patients were 0.81 (95%CI: 0.42-1.56) for all bleeding and 0.76 (95%CI: 0.37-1.54) for minor bleeding complications. Numbers were too low to calculate an adjusted relative risk for major bleeding complications in patients on phenprocoumon. During the initiation phase of therapy, we found no effect of variant genotype on bleeding risk, neither for all bleeding (RR 1.00, 95% CI: 0.62-1.61), nor for minor (RR 1.02, 95%CI: 0.61-1.69), and major bleeding events (RR 0.82, 95%CI: 0.21-3.19). For carriers of a CYP2C9*2 allele, relative risks for all bleeding (RR 1.11, 95%CI: 0.83-1.50), minor bleeding (RR 1.02, 95%CI: 0.73-1.43) and major bleeding events (RR 1.60, 95%CI: 0.85-3.04) were more or less the same as the hazards ratios for the combined variant genotypes. Relative risks for carriers of a CYP2C9*3 allele were 0.69

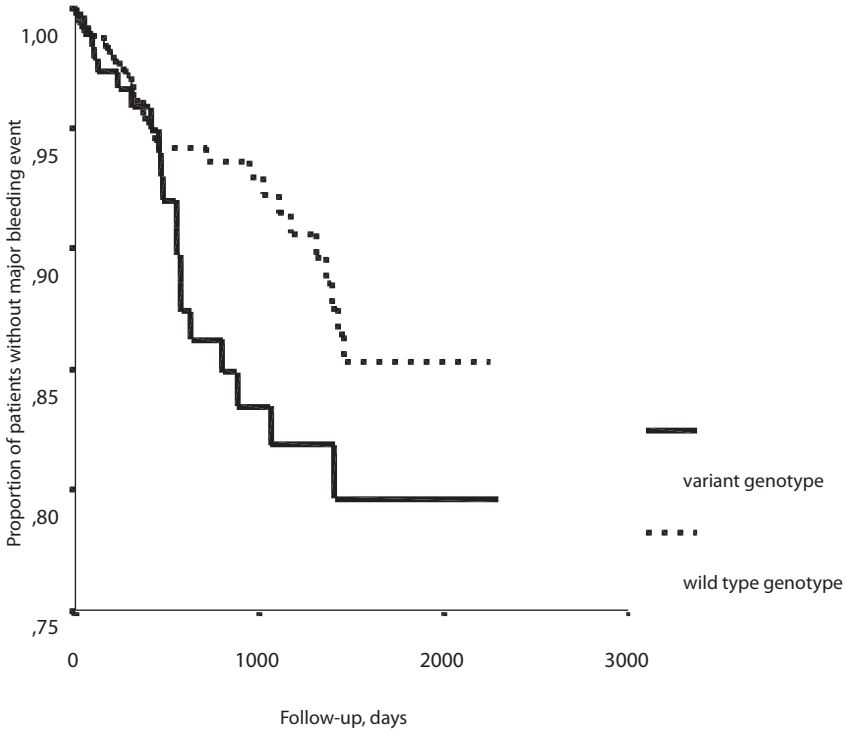

Figure 1. Proportion of patients without major bleeding event.

(95%CI: 0.42-1.15) for all bleeding, 0.49 (95%CI: 0.26-0.94) for minor bleeding and 1.69 (95%CI: 0.72-3.93) for major bleeding complications.

DISCUSSION

The main finding in this population-based cohort study is that having a variant allele of CYP2C9 was associated with an increased risk of major bleeding events in patients on acenocoumarol. The fact that we did not find such an association for phenprocoumon is probably explained by the fact that the CYP2C9 enzyme plays a minor role in its metabolism [8]. This is in line with an earlier study in which 37 out of 262 carriers of a variant allele and 67 out of 579 patients with the wild type genotype experienced an INR $\geq$ 6.0 [29]. In this study CYP2C9 variant alleles were associated with an increased risk of overanticoagulation in users of acenocoumarol but not in users of phenprocoumon [29]. It should be emphasized, however, that the number of patients on phenprocoumon in the current study was relatively small. Nevertheless, this aspect may be a reason to prefer phenprocoumon over acenocoumarol. In our study, CYP2C9 genotype was not associated with a higher rate of bleeding events during the first 90 days of therapy. A higher frequency of bleeding early in the treatment course has been reported in many [13, 14,

16, 23-26] but not all [18, 27, 28] studies. As clearly pointed out by Landefeld and Goldman [25], studies that examine non-inception cohorts are likely to underestimate the true risk of bleeding by missing early events. We tried to reduce this selection bias by excluding prevalent users at the time the study started. Moreover, in a different study with an INR ≥ 6.0 as an outcome, [29] we did find a small but statistically significantly increased risk of overanticoagulation during the initiation phase in patients with CYP2C9 variant alleles on acenocoumarol. Forty out of 301 carriers of a variant allele and 58 out of 669 patients with the wild type genotype experienced an INR ≥ 6.0 (RR 1.5, 95%CI: 1.1-2.2). These apparently different results during the initiation phase of therapy are possibly due to the fact that an INR ≥ 6.0 is a more frequent outcome than bleeding, which therefore has a higher chance to occur early after starting treatment. Because severe bleeding is much rarer, a longer period may be required to demonstrate a significant risk difference between persons with variant alleles and those with the wild type. Moreover, after administration of loading doses that are apparently too high for carriers of variant alleles because their INR increases too strongly, clinicians probably make rapid, downward dose adjustments before the bleeding risk is significantly increased. Our results are in line with Tassies et al [18], who found that carriers of the CYP2C9*3 allele on acenocoumarol were more prone to suffer overanticoagulation at the initiation of therapy. Also in that study, however, this was not associated with a higher rate of bleeding.

Reliable data are lacking on the true frequency of complications in patients on coumarin anticoagulants because of methodological limitations [30]. The incidences of minor bleeding events in our study are similar to those found in other observational studies [5, 22, 30]. The major bleeding rate of 4.0 per 100 treatment-years is somewhat lower than the 4.9 per 100 treatment-years reported in a review of observational studies [22], but higher than the 2.7 [5] and 2.1 per 100 treatment-years reported by Van der Meer [30]. The studies reported by Van der Meer, however, were performed in a similar setting and with the same definitions of bleeding complications. The differences can possibly be explained by the fact that we used more data sources for case finding than the data from the anticoagulation clinic. In our study, major bleeding complications were not always reported in the database from the anticoagulation clinic. Usage of only this data would have resulted in lower incidence rates. Another possible explanation is that Van der Meer did not examine inception cohorts but also included prevalent users of anticoagulant therapy. In our study, the higher risk in patients with variant alleles on acenocoumarol was only found for major and fatal bleeding events but not for minor events. A possible explanation for this difference might be that patients are asked whether they had a bleeding event every time they visit the monitoring center. This will probably lead to an overrepresentation of bleeding, including false-positives and trivial cases. Such misclassification will be random for genotypes. It is known that random misclassification introduces a bias towards the null hypothesis. As severe bleeding is acute and unexpected, such misclassification does not occur.

The allele frequency for CYP2C9*2 found in our study population falls within the wide range of reported allele frequencies among various Caucasian populations (8-19%) and is very close to those found in other Dutch, German and British populations [31]. Caucasians also exhibit a significant heterogeneity in CYP2C9*3 allele frequency (3.3-16.2%). Whereas the allele frequency for CYP2C9*3 in our population falls within this range, it is however lower than the ones reported in other northern European countries [31]. The study population is at Hardy-Weinberg equilibrium for overall variant alleles. For phenotype prediction, variant alleles CYP2C9*2 and CYP2C9*3 can be taken together because there is no phenotype/genotype disagreement in the metabolism of coumarin anticoagulants for these variant alleles [32].

In conclusion, having a variant allele of CYP2C9 was associated with an increased risk of major bleeding events in patients on acenocoumarol, but not in patients on phenprocoumon. Although one might consider the assessment of the CYP2C9 genotype of a patient for dose adjustment before starting treatment with acenocoumarol, a prospective randomised trial should demonstrate whether this reduces the increased risk of major bleeding events.

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Chapter 2.3

The risk of myocardial infarction in patients with reduced enzyme activity of cytochrome P450 CYP2C9

ABSTRACT

Evidence suggests that certain human cytochrome P450 enzymes are partly responsible for the metabolism of endogenous substances involved in the regulation of vascular homeostasis. Genetic polymorphisms that code for these enzymes or use of drugs that inhibit these enzymes may therefore have pathophysiological consequences for cardiovascular diseases such as myocardial infarction. We investigated whether the variant alleles CYP2C9*2 and CYP2C9*3 or use of CYP2C9 substrates were associated with an increased risk of myocardial infarction in 2210 men and 3534 women from the Rotterdam Study, a prospective population-based cohort study of individuals aged 55 years or older. In women, use of CYP2C9 substrates was significantly associated with incident myocardial infarction (RR 2.68, 95%CI: 1.60-4.49). The risk of myocardial infarction was fivefold in female users of CYP2C9 substrates with a variant allele. Neither the use of CYP2C9 substrates, nor the variant alleles were associated with an increased risk of myocardial infarction in men.

INTRODUCTION

Myocardial infarction is a complex multifactorial and polygenic disorder that is thought to result from an interaction between the genetic makeup of an individual and various environmental factors [1, 2]. In general, the incidence of myocardial infarction increases with the number of conventional risk factors, including hypertension, diabetes mellitus, and hypercholesterolemia [2]. Although each risk factor itself is partly under genetic control, a family history of myocardial infarction is also an independent predictor. This suggests that additional susceptibility genes play an important role in this condition [1]. There is evidence that the human cytochrome P450 enzymes CYP2C8 and CYP2C9 are partly responsible for the metabolism of endogenous substances involved in the regulation of vascular homeostasis, notably the arachidonic acid metabolites 5,6-; 8,9-; 11,12-; and 14,15-epoxyeicosatrienoic acids (EETs) [3-7]. These EETs play a prominent role as a nitrous oxide/prostacyclin-independent component of endothelium-dependent relaxation, which is particularly prominent in coronary, mesenteric, and renal arteries [3-5, 8, 9]. This system seems to have a more prominent role in females than in males [10, 11]. Polymorphisms in CYP2C8 and CYP2C9 enzymes may therefore have important pathophysiological consequences in cardiovascular diseases such as myocardial infarction. The CYP2C enzymes are predominantly expressed in the liver but also in the vascular smooth muscles and endothelial cells [3-5, 12, 13]. In a recent study, it was hypothesized that allelic variants of the CYP2C8 and CYP2C9 genes were associated with a modest increase in risk of myocardial infarction in women [14]. Apart from this genetic component, it is conceivable, that drugs that are metabolized by CYP2C9 can also affect levels of EETs, because several of these inhibit CYP2C9 enzymes.

Therefore, the aim of the present follow-up study was to investigate whether the variant alleles CYP2C9*2 and CYP2C9*3 were associated with an increased risk of myocardial infarction in men and women. Furthermore, we determined the risk of myocardial infarction in male and female users of drugs that are metabolized by CYP2C9 and studied whether there was effect modification by CYP2C9 genotype.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study of neurological, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, aged 55 years or older were invited in 1990-1993 to participate in the study. The Medical Ethics Committee of the Erasmus MC approved the study and written informed consent was obtained from all participants. The rationale and design of this study have been described elsewhere [15]. The cohort encompasses 7983 individuals

who were all interviewed and investigated at baseline. Since the start of the Rotterdam Study, cross-sectional surveys have been carried out periodically. In addition, participants are continuously monitored for major events through automated linkage with the files from the general practitioners of the patients. When an event or death has been reported, additional information is obtained by interviewing the GP and by scrutinising information from hospital discharge records in case of admittance or referral. More than 99% of participants have their drug prescriptions filled at 7 regional pharmacies, which are fully computerized. Complete data on drug use were available as of January 1, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC) code [16], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and the product name of the drugs.

Cohort definition

For the present study, we included all patients in the Rotterdam Study from whom a blood sample was taken, whose CYP2C9 status was analyzed and who did not have a history of myocardial infarction as based on a patient's interview, medical records from general practitioner or cardiologists, electrocardiography, and data on all hospital admissions. The cohort was followed until the first occurrence of a myocardial infarction, death or end of the study period, whichever came first. Follow-up started at the baseline examination and is continuously performed. For the present study, we used all data up to January 1, 2000.

Outcome

Myocardial infarction was classified as present when a hospital discharge diagnosis of myocardial infarction was present, or, in case a patient was not hospitalised, when signs and symptoms, ECG recordings and cardiac enzyme data were diagnostic of myocardial infarction [17]. Two research physicians independently coded the possible cardiac events, according to the International Classification of Diseases, 10th version [18]. A cardiologist reviewed the coded events and performed the definitive coding. Only definite and probable cases were included in the analyses. The date on which an incident myocardial infarction was encountered was defined as the index date.

Genotyping

Genotyping for the CYP2C9*2 and CYP2C9*3 allele variants was performed by using polymerase chain reaction followed by restriction enzyme digestion analysis (PCR-RFLP), as previously described by Aynacioglu et al [19]. All CYP2C9*2 and CYP2C9*3 heterozygote and homozygote variants detected were reanalyzed. Patients in whom neither CYP2C9*2 nor CYP2C9*3 alleles were identified were regarded as wild type.

Drug exposure

We assessed exposure to the following CYP2C9 substrates and inhibitors (CYP2C9 drugs) on the index date (http://www.hcuge.ch/pharmacie/listemed/index.htm): aceclofenac, acenocoumarol, amiodarone, carbamazepine, celecoxib, chloroquine, clopidogrel, clozapine, cyclophosphamide, delavirdine, desogestrel, diclofenac, efavirenz, fluconazole, fluoxetine, flurbiprofen, fluvastatin, fluvoxamine, gemfibrozil, ibuprofen, imatinib, indometacin, irbesartan, losartan, mefenamic acide, meloxicam, metronidazole, miconazole, montelukast, naproxen, nateglinide, phenobarbital, phenprocoumon, phenylbutazone, phenytoin, pioglitazone, piroxicam, propofol, ritonavir, rosiglitazone, sildenafil, simvastatin, sulfamethoxazole, tamoxifen, tenoxicam, terbinafine, torasemide, valproic acid, verapamil, and zafirlukast. In order to prevent confounding by indication we excluded the following cardiovascular drugs: acenocoumarol, amiodarone, clopidogrel, fluvastatin, gemfibrozil, irbesartan, losartan, phenprocoumon, simvastatin, torasemide and verapamil.

Potential confounders and effect modifiers

Information was gathered at baseline on several potential confounders and effect modifiers such as age, gender, ethnic origin, body mass index (kg/m^2) and smoking (classified as never/former/current). At the research center, non-fasting blood samples were taken and serum total cholesterol and high-density lipoprotein (HDL) cholesterol was measured using an automated enzymatic procedure. Systolic and diastolic blood pressures from the right upper arm were measured twice with a random-zero sphygmomanometer with the patient in a sitting position. The mean of the two readings was used to determine blood pressure levels. Hypertension was defined as use of hypertensive medication for the indication high blood pressure, or as a systolic blood pressure of 140 mm Hg or over, or a diastolic blood pressure of 90 mm Hg or over [20]. Diabetes mellitus was considered present on the basis of use of antidiabetic medication, or a random or post-load glucose level higher than 11.0 mmol/l. Hypercholesterolemia was defined as use of lipid lowering medication or a serum total cholesterol higher than 6.5 mmol/l. Intima-media thickness (IMT) of the carotid arteries, measured by ultrasonography, was used as a measure of atherosclerosis. The maximum common carotid IMT was determined as the average of the maximum IMT of near- and far-wall measurements, and the average of left and right maximum common carotid IMT was computed [21]. To indicate no, mild, moderate, and severe thickening of the carotid wall, we divided the IMT into gender-specific quartiles.

Statistical analysis

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium (HWE) by using a χ^2-test.

The association between CYP2C9 genotype and myocardial infarction was evaluated using survival analysis techniques, for men and women separately. The reason for these a priori stratified analyses was that EET-induced vasodilatation is suggested to be more important

in females than in males. Patients were divided into 2 groups based on genotype: wild type (CYP2C9*1/*1 homozygotes) and variant (1 or 2 mutant alleles of CYP2C9*2, or CYP2C9*3). For each analysis, a relative risk (RR) and 95% confidence interval (95%CI) comparing variant and wild type genotype were computed. We used Cox proportional hazards models to adjust for the potential confounding effect of age, and additionally for cardiovascular risk factors, including body mass index, smoking, hypertension, diabetes mellitus, hypercholesterolemia, HDL cholesterol, and atherosclerosis. We tested for interaction between CYP2C9 genotype and atherosclerosis by adding an interaction term to the regression model: CYP2C9 variant genotype (0-1) x carotid IMT (1-4).

Cox proportional hazards regression for time-dependent variables was used to compute relative risks of myocardial infarction in male and female users of CYP2C9 drugs compared to non-users [22]. The multivariate model was adjusted for the abovementioned cardiovascular risk factors as well as for CYP2C9 genotype. We tested for interaction between the use of CYP2C9 drugs and CYP2C9 genotype by adding an interaction term to the regression model: use of CYP2C9 drugs (0-1) x CYP2C9 variant genotype (0-1).

For categorical covariates with missing values we incorporated missing indicator variables in the model. For subjects with missing data on covariates measured on a continuous scale, we imputed the gender-specific population mean. A P-value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS software (version 11.0.1; SPSS Inc., Chicago, USA).

RESULTS

From the initial 7983 participants of the Rotterdam Study, 1376 were excluded because a blood sample had never been drawn or no material was left for CYP2C9 genotyping. From the 6607 remaining individuals, 92 subjects (1.4%) were excluded because of difficulties with genotyping. Subjects who experienced a myocardial infarction before the baseline examination (n = 771) were excluded. Consequently, there were 5744 individuals (2210 men and 3534 women) available for analysis. All patients were of Caucasian origin. There were 3808 patients (66.3%) with the wild type genotype, and 1936 (33.7%) with a variant genotype. The frequencies of the CYP2C9*2 and CYP2C9*3 alleles were 12.8% and 5.8%, respectively. Allele and genotype distributions were in Hardy-Weinberg equilibrium and frequencies were similar to other studies of Caucasian subjects [23]. The mean follow-up time of the study population was 7.0 years (standard deviation 2.1 years; range 18 days to 10.5 years). During follow-up, incident myocardial infarction occurred in 128 men and 95 women. Table 1 shows the baseline characteristics of the incident cases of myocardial infarction and the total cohort. As expected, for both men and women, incident cases of myocardial infarction had a more adverse cardiovascular risk profile than the total cohort.

Table 1. Baseline characteristics in men and women from the Rotterdam Study

Characteristic*	Men (N = 2210)			Women (N = 3534)		
	Patients with MI (N = 128)	Cohort (N = 2210)	P	Patients with MI (N = 95)	Cohort (N = 3534)	P
Age, years	69.2 ± 7.2	67.8 ± 8.2	0.001	72.5 ± 8.7	69.9 ± 9.5	<0.001
Body mass index (kg/m^2)	25.7 ± 2.7	25.6 ± 2.9	0.958	27.5 ± 3.7	26.7 ± 3.9	0.050
Smoking (% current)	24.2	29.7	0.656	23.2	17.6	0.241
Hypertension (%)	57.8	47.6	0.016	64.2	51.3	0.001
Diabetes mellitus (%)	11.7	8.8	0.112	17.9	10.0	0.002
Hypercholesterolemia (%)	53.1	40.0	0.006	78.9	59.8	<0.001
HDL-cholesterol (mmol/L)	1.15 ± 0.24	1.23 ± 0.33	0.005	1.29 ± 0.37	1.44 ± 0.37	<0.001
Intima-media thickness (mm)	0.87 ± 0.14	0.84 ± 0.13	0.003	0.87 ± 0.14	0.81 ± 0.12	<0.001
Genotype			0.195†			0.183†
CYP2C9*1/*1 (%)	87 (68.0)	1473 (66.7)		58 (61.1)	2335 (66.1)	
CYP2C9*1/*2 (%)	27 (21.1)	457 (20.7)		23 (24.2)	744 (21.1)	
CYP2C9*1/*3 (%)	12 (9.4)	211 (9.5)		9 (9.5)	323 (9.1)	
CYP2C9*2/*2 (%)	1 (0.8)	39 (1.8)		4 (4.2)	53 (1.5)	
CYP2C9*2/*3 (%)	1 (0.8)	21 (1.0)		1 (1.1)	61 (1.7)	
CYP2C9*3/*3 (%)	0	9 (0.4)		0	18 (0.5)	

*Values of continous variables are expressed as mean ± standard deviation. Categorical variables are expressed as percentage.
† P-value for Hardy Weinberg Equilibrium.

Table 2 shows that variant CYP2C9 genotype was not associated with myocardial infarction in men (RR 0.98, 95%CI: 0.68-1.42). For women, the adjusted relative risk was somewhat higher (RR 1.21, 95%CI: 0.80-1.82), but also not statistically significant. The risk estimates did not change after adjusting for clinically relevant cardiovascular risk factors. For both men and women, atherosclerosis did not modify the association between variant CYP2C9 genotype and incident myocardial infarction.

Table 3 shows that the risk of myocardial infarction was statistically significantly increased in female users of CYP2C9 drugs (RR 2.68, 95%CI: 1.60-4.49), but not in male users (RR 1.26, 95%CI: 0.64-2.49). The interaction term between use of CYP2C9 drugs and CYP2C9 genotype was

Table 2. Relative risks for incident myocardial infarction associated with CYP2C9 genotype for men and women

	Number	Events	Model 1* RR (95%CI)	Model 2† RR (95%CI)
Men				
Wild type‡	1473	87	1.00 (reference)	1.00 (reference)
Variant genotype§	737	41	0.96 (0.67-1.40)	0.98 (0.68-1.42)
Women				
Wild type‡	2335	58	1.00 (reference)	1.00 (reference)
Variant genotype§	1199	37	1.25 (0.83-1.88)	1.21 (0.80-1.82)

* Model 1: adjusted for age.
† Model 2: adjusted for age, body mass index, smoking, hypertension, diabetes mellitus, hypercholesterolemia, HDL cholesterol and carotid IMT.
‡ CYP2C9*1/*1 homozygotes.
§ Patients with one or more of the variant alleles CYP2C9*2 or CYP2C9*3.

Table 3. Relative risks for incident myocardial infarction associated with use of CYP2C9 drugs* and effect modification by CYP2C9 genotype for men and women

	Number†	Events‡	Model 1§ RR (95%CI)	Model 2** RR (95%CI)
Men				
No use of CYP2C9 drugs	340315	107	1.00 (reference)	1.00 (reference)
Use of CYP2C9 drugs	20829	9	1.35 (0.69-2.67)	1.26 (0.64-2.49)
*P-value for statistical interaction CYP2C9 drugs * CYP2C9 genotype*	*0.398*			
Wild type; no use of CYP2C9 drugs	227082	70	1.00 (reference)	1.00 (reference) ††
Wild type; use of CYP2C9 drugs	13534	7	1.64 (0.75-3.56)	1.54 (0.70-3.35)††
Variant genotype; no use of CYP2C9 drugs	113233	37	1.07 (0.72-1.59)	1.06 (0.71-1.58) ††
Variant genotype; use of CYP2C9 drugs	7295	2	0.89 (0.22-3.64)	0.85 (0.21-3.48) ††
Women				
No use of CYP2C9 drugs	548916	64	1.00 (reference)	1.00 (reference)
Use of CYP2C9 drugs	57732	19	2.79 (1.67-4.68)	2.68 (1.60-4.49)
*P-value for statistical interaction CYP2C9 drugs * CYP2C9 genotype*	*0.084*			
Wild type; no use of CYP2C9 drugs	361749	40	1.00 (reference)	1.00 (reference) ††
Wild type; use of CYP2C9 drugs	38727	8	1.88 (0.88-4.05)	1.80 (0.84-3.86)††
Variant genotype; no use of CYP2C9 drugs	187167	24	1.16 (0.70-1.92)	1.14 (0.69-1.89) ††
Variant genotype; use of CYP2C9 drugs	19005	11	5.28 (2.69-10.36)	5.00 (2.55-9.83) ††

* Definition of CYP2C9 drugs: see text.
† In this time-dependent analysis, exposure in case patients and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). Because control patients can be used multiple times, the number of assessments is much larger than the number of individuals. Hence, crude relative risks cannot be calculated with the number in this table.
‡ 12 events in male and 12 events in female users of cardiovascular drugs excluded to prevent confounding by indication.
§ Model 1: adjusted for age.
** Model 2: adjusted for age, body mass index, smoking, hypertension, diabetes mellitus, hypercholesterolemia, HDL cholesterol, carotid IMT, and CYP2C9 genotype.
†† Model 2: adjusted for age, body mass index, smoking, hypertension, diabetes mellitus, hypercholesterolemia, HDL cholesterol, and carotid IMT.

not statistically significant in male users of CYP2C9 drugs (p = 0.398), and almost statistically significant in female users (p = 0.084). The adjusted relative risk of myocardial infarction in female users of CYP2C9 drugs with a variant genotype was 5.00 (95%CI: 2.55-9.83) as compared to women with the wild type genotype who did not use CYP2C9 drugs.

DISCUSSION

We found that women who used drugs that are metabolized by CYP2C9 had an increased risk of myocardial infarction, and that in women with allelic variants of CYP2C9 this risk was even higher. A potential explanation might be that this is the consequence of impaired formation of endogenous CYP2C9-derived vasoactive EETs, due to the concomitant inhibition of the enzyme by the CYP2C9 drugs and the decreased enzyme activity in patients with allelic

variants. Neither the use of CYP2C9 drugs alone, nor a variant genotype was associated with a significantly increased risk of myocardial infarction in men. These findings are in accordance with the study of Yasar et al, who found an increased risk in women but not in men [14]. The reason for this gender-based difference remains unclear but might be attributable, at least in part, to the differences in the levels of estrogen or other hormones between men and women. Population-based studies suggest that before menopause women have a delayed and less severe manifestation of cardiovascular diseases than men [24, 25]. This gender difference is thought to be due to an estrogen-stimulated enhanced capacity of endothelial cells of arterioles from females to produce nitric oxide [10]. These effects on vascular NO production require the chronic presence of estrogen [10] and are therefore probably diminished after menopause. Evidence suggests that important differences exist in the adaptation of the endothelium of arterioles from male and female rats to the lack of nitric oxide synthesis [11]. An augmented release of endothelial prostaglandins accounts for the preserved flow-induced dilation in arterioles of male rats, whereas a CYP-derived metabolite is responsible for the maintenance of flow-induced dilation in female rats [11]. Inhibition of this compensatory mechanism by use of CYP2C9-inhibiting drugs could therefore be the cause of the increased risk of incident myocardial infarction in postmenopausal women and the reason why we did not find such an association in men. The reason for the larger impact of drugs metabolized via CYP2C9 on the risk of myocardial infarction, as compared to the effect of the variant alleles CYP2C9*2 and CYP2C9*3, could be that the cardiovascular system is adapted to this permanently reduced enzyme activity in the case of the variant alleles and myocardial infarction mainly occurs when the CYP2C9 enzyme is suddenly blocked by use of CYP2C9 drugs.

Selection bias was negligible because our study population comprised all men and women in the Rotterdam Study. Furthermore, our study population was in Hardy-Weinberg equilibrium, suggesting that no selection has occurred among genotypes, which could have explained the observed association. Also, information bias is not likely, since all data on CYP2C9 genotype and myocardial infarction were recorded similarly for all participants without prior knowledge of our study hypothesis. Random misclassification of the outcome may have occurred due to measurement error but is unlikely as myocardial infarction is a diagnosis, which is usually fairly specific. Moreover, any random misclassification would tend to underestimate rather than overestimate the true risk. Potential confounding factors were dealt with in the analyses. Although we had no data on the presence and severity of coronary atherosclerosis, the extracoronary measure of atherosclerosis we used, has been demonstrated to be a reliable indicator of atherosclerosis because of its consistent associations with atherosclerosis in other arteries, cardiovascular risk factors, and future cardiovascular diseases [26]. As in all pharmaco-epidemiological studies, confounding-by-indication is a potential problem. Therefore, we excluded all CYP2C9 drugs with the indication cardiovascular disease.

Although 12 different single nucleotide polymorphisms (SNPs) have been described in the coding region of the CYP2C9 gene (http://www.imm.ki.se/CYPalleles), we only analysed the

CYP2C9*1, *2, and *3 alleles, because these were the alleles reported in Caucasian populations [23]. Yasar et al [27] recently reported on a strong, but not complete, linkage between the CYP2C8*3 and CYP2C9*2 allelic variants. The CYP2C8*3 variant, with an allele frequency of 13% in Caucasians, was associated with a markedly defective metabolism of arachidonic acid, corresponding to 35% of the CYP2C8*1 activity. It was speculated that for some substrates that are metabolised by both CYP2C8 and CYP2C9, like arachidonic acid, an impaired clearance in vivo previously exclusively attributed to the CYP2C9*2 variant could in part be explained by slow metabolism of the substrate by the associated CYP2C8*3 variant. The samples in the present study were not analysed for the CYP2C8*3 allele. It is conceivable that this variant could have had a risk modifying effect of the CYP2C9 gene on myocardial infarction.

In conclusion, this population-based cohort study shows a significant almost three-fold increased risk of incident myocardial infarction in women who used drugs that are metabolized by CYP2C9. This risk is even further increased in women with a variant allele.

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Chapter 2.4

Patients with an ApoE ε4 allele require lower doses of coumarin anticoagulants

ABSTRACT

Objective: Vitamin K is an essential cofactor for the synthesis of several blood coagulation factors. It has been suggested that the ApoE genotype has profound effects on vitamin K status. Therefore, we investigated whether this common genetic polymorphism influenced dose requirements and effects of coumarin anticoagulants.

Methods: We did a cohort study in 1637 patients from an outpatient anticoagulation clinic treated with acenocoumarol or phenprocoumon.

Results: To attain the same level of anticoagulation, patients with genotype ε4/ε4 and genotype ε3/ε4 required respectively 3.4 mg (95%CI: -6.0 to -0.9) and 0.8 mg (95%CI: -1.6 to 0.1) acenocoumarol per week less than patients with genotype ε3/ε3. Patients homozygous for the ε2 allele required 3.5 mg (95%CI: 0.1 to 6.9) acenocoumarol per week more than patients with genotype ε3/ε3. The effect of the ε4 allele on the acenocoumarol maintenance dose was dose-dependent, while the effect of the ε2 allele was not. No significant dose difference was observed for phenprocoumon, possibly because of low numbers.

Conclusion: The ApoE genotype affects the dose requirements of acenocoumarol.

INTRODUCTION

Vitamin K is an essential cofactor for carboxylation of the blood coagulation factors II, VII, IX, X, and several other calcium-binding proteins [1-3]. Coumarin anticoagulants inhibit vitamin K regeneration in the liver, and thereby reduce the synthesis of carboxylated blood coagulation factors [4]. Vitamin K from dietary or intestinal sources directly counteracts the effect of coumarin anticoagulants. The major dietary and circulating form of vitamin K is phylloquinone (vitamin K_1) [5]. In plasma, vitamin K_1 is bound to chylomicrons and chylomicron remnants [6]. The uptake of these vitamin K-rich lipoproteins by liver and other tissues is mediated by apolipoprotein E (ApoE), a constituent of chylomicron remnants, which binds to lipoprotein receptors [7]. ApoE is a polymorphic protein, defined by three alleles ε2, ε3, and ε4 at a single gene locus on chromosome 19. These alleles code for three isoforms of ApoE (E2, E3, and E4) that differ by one or both of two amino acid substitutions at sites 112 and 158, and thus determine the phenotype of the six genotypes resulting from the combination of any two of these alleles [8]. Chylomicron-remnant clearance is thought to be slowest in people with genotype ε2/ε2, faster in those with genotype ε3/ε3, and fastest in those with genotype ε4/ε4. Individuals with the heterozygous genotypes were reported to have clearance rates that are intermediate to those with the respective homozygous genotypes [9]. As a consequence, plasma vitamin K_1 levels are strongly influenced by ApoE genotype, being highest in those with the E2 isoform, intermediate in E3 and lowest in those with the E4 isoform [6,10,11]. The association between ApoE genotype and plasma vitamin K_1 levels raise the possibility that the sensitivity to coumarin anticoagulants is also partly determined by ApoE genotype.

Therefore, we investigated the effect of the ApoE genotype on the maintenance dose of acenocoumarol and phenprocoumon, on the International Normalised Ratio (INR), a standardized measure of anticoagulation, and on bleeding complications.

METHODS

Setting

Data were obtained from a regional outpatient anticoagulation clinic and from the Rotterdam Study. The anticoagulation clinic monitors all inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. Almost all patients start with a standard dosing scheme of acenocoumarol (8-4-4 mg during day 1 up to day 3) or phenprocoumon (9-6-3 mg during day 1 up to day 3). The optimal target range of coumarin anticoagulant therapy, as recommended by the Federation of Dutch Thrombosis Centers, lies between 2.5 and 3.5 INR or between 3.0 and 4.0 INR, depending on the indication for treatment. Some patients are targeted at a level between 2.0 and 2.5 INR because of contraindications. Prothrombin times are monitored each

one to six weeks by reference to the INR, dependent on the stability of the anticoagulant level. Doses are adjusted on the basis of the target range of the INR of the patient by computerised dose calculations. All data on dosing, laboratory-, and clinical data (e.g. bleeding complications) as of 1984 are fully computerised. For this study, data were used from January 1, 1985 through May 31, 2003. The Rotterdam Study is a prospective population-based cohort study of neurological, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants aged 55 years or older in Ommoord were invited in 1990-1993 to participate in the study. The rationale, ethical approval and design of this study have been described elsewhere [12]. The Rotterdam Study encompasses 7,983 individuals who were all interviewed and investigated at baseline.

Cohort definition

The study cohort consisted of all 1983 patients from the anticoagulation clinic, who started treatment with acenocoumarol or phenprocoumon in the study period between January 1, 1985 and May 31, 2003, for whom INR data from their treatment history were available, and whose ApoE status was analysed in the Rotterdam Study. If a patient had multiple treatment episodes during the study period, all episodes were considered. We defined the follow-up period as the sum of all treatment episodes per patient. The cohort was followed until the last INR-assessment because of the end of their treatment, death or end of the study period, whichever came first.

Outcomes and confounders

The average first INR following the standard start-dosing scheme of acenocoumarol or phenprocoumon was calculated per ApoE genotype. Furthermore, the occurrence of overanticoagulation during the first six weeks of treatment and during the whole follow-up period was assessed. Overanticoagulation was defined as an INR $\geq$ 6.0, since at this INR-value the risk of bleeding sharply increases [13]. Patients, who had a follow-up period of more than six weeks, were included in the analyses on maintenance dose. The maintenance dose was defined as the mean dose (in mg per week) calculated over all treatment episodes from day 43 up to the end of each treatment episode. The mean INR was calculated over the same period. As a clinical outcome, we included all bleeding complications, including skin bleeds of more than 10 cm in diameter and nosebleeds lasting for at least 30 minutes.

As potential confounders we considered: age, gender, target INR level, measured INR, cytochrome P450 2C9 (CYP2C9) genotype, and use of statins. In the analyses on overanticoagulation and bleeding complications in relation to variant genotype, the mean time between the INR measurements was also taken into account.

Genotyping

DNA was used for ApoE genotyping [14]. The ApoE gene was amplified by the primer and amplification conditions described by Wenham and colleagues [15]. After amplification, the

PCR product was digested with the restriction enzyme HhaI and fragments were separated by electrophoresis on a 5% agarose gel. ApoE alleles were visualized by staining with ethidium bromide. We defined ε4 carriers as subjects with ε3/ε4, or ε4/ε4 alleles and ε2 carriers as subjects with ε2/ε3, or ε2/ε2 alleles. Hence, the ε2/ε4 genotype was excluded from these analyses to facilitate mutually exclusive categories. Subjects with genotype ε3/ε3 were regarded as the reference group.

Statistical analysis

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium (HWE) by using a χ^2-test. Independent-samples t tests were used for comparing the mean first INR after a standard start dose, the mean INR and the mean maintenance dose, between all variant genotypes and the ε3/ε3 genotype, and between ε2 or ε4 carriers and the reference genotype. The associations between ApoE genotype and overanticoagulation and between ApoE genotype and bleeding complications were evaluated using survival analysis techniques. For these analyses, patients were divided into 2 groups based on genotype: variant genotype (ε2/ε2, ε2/ε3, ε3/ε4, ε2/ε4, ε4/ε4) and reference genotype (ε3/ε3). For each analysis, a hazard ratio (HR) and 95% confidence interval (95%CI) comparing variant and reference genotype, were computed. These analyses were performed with SPSS software (version 10.0; SPSS, Chicago, IL). Then, ApoE genotype was examined as a determinant of coumarin dose over time. To analyse correlated data (repeated INR measurements and the subsequent doses in the same person), unbalanced repeated measurement analysis [16,17] was used with the Proc Mixed module of SAS (version 8.2). The general linear mixed model included coumarin dose as the outcome variable and ApoE genotype as well as age, gender, target INR, measured INR, CYP2C9 genotype, and use of statins as explanatory variables. To evaluate an allele-dose effect for the ε2 allele and for the ε4 allele, trend tests were performed using the same linear model with the mean maintenance dose as outcome variable and the allele dose as an ordinal set with 3 values (0, 1 or 2 copies of the test allele). We tested for interaction between the ApoE ε4 allele and CYP2C9 variant genotype by adding an interaction term to the model: CYP2C9 variant genotype (0-1) x ApoE genotype (0-1-2: non carriers-ε4 heterozygotes-ε4 homozygotes), assuming an allele-effect relationship. P values less than 0.05 were considered statistically significant.

RESULTS

Of the 1983 patients in the cohort, 346 patients were excluded because of missing ApoE genotypes. Consequently, there were 1637 patients available for analysis (Table 1). The mean age of these patients was almost 75 years, and 44.3% of the patients were men. All patients were of Caucasian origin. There were 1490 acenocoumarol-treated patients (91.0%), and 220 patients (13.4%) who used phenprocoumon. Seventy-six of them used both coumarins during

Table 1. Characteristics of the study population

Variable	Number of patients (n = 1637)
Age, average (SD)	74.8 (8.1) years
Gender	
Male	725 (44.3%)
Female	912 (55.7%)
Caucasian origin	1637 (100%)
Type of coumarin*	
Acenocoumarol	1490 (91.0%)
Phenprocoumon	220 (13.4%)
Unknown	3 (0.2%)
Target INR level	
Very low (2.0-2.5)	54 (3.3%)
Low (2.5-3.5)	765 (46.7%)
Normal (3.0-4.0)	818 (50.0%)
Weekly maintenance dose range	
Acenocoumarol	1 - 64 mg
Phenprocoumon	1 - 58 mg
Follow-up time	
Mean	747 days
Median	238 days
ApoE genotype	
ε2/ε2	16 (1.0%)
ε2/ε3	200 (12.2%)
ε3/ε3	943 (57.6%)
ε3/ε4	397 (24.3%)
ε2/ε4	49 (3.0%)
ε4/ε4	32 (2.0%)
CYP2C9 genotype	
*1/*1	1021 (62.4%)
*1/*2	342 (20.9%)
*2/*2	31 (1.9%)
*1/*3	148 (9.0%)
*2/*3	29 (1.8%)
*3/*3	2 (0.1%)

* Because 76 patients used both coumarins at any time during the study period, the total percentage is more than 100.

the study period. Fifty-four patients (3.3%) were targeted at an INR between 2.0 and 2.5, 765 patients (46.7%) at an INR between 2.5 and 3.5, and 818 patients (50.0%) at an INR between 3.0 and 4.0. The weekly maintenance dose of acenocoumarol ranged from 1 to 64 mg and of phenprocoumon from 1 to 58 mg. The mean follow-up time was 747 days (2.0 years). Patients had a median of 20 INR assessments during a median follow-up time of 238 days (0.7 years). Table 1 shows the distribution of patients by ApoE genotype. There were 216 ε2 carriers (ε2/ε4 genotype excluded) and 429 ε4 carriers. The allele frequencies of the ε2, ε3 and ε4 alleles were 0.086, 0.758 and 0.156 respectively. There was no significant departure of genotype - and

Table 2. The mean first INR after a standard start dose, the mean INR and the mean maintenance dose of acenocoumarol and phenprocoumon in relation to ApoE genotype

Genotype	N pat	Mean first INR (SEM*)	p-value†	Mean INR (SEM*)	p-value‡	Mean maintenance dose (mg/week) (SEM*)	p-value§
Acenocoumarol	1490						
ε2/ε2	14	2.79 (0.39)	0.99	3.40 (0.04)	<0.001	17.8 (0.33)	<0.001
ε2/ε3	174	2.85 (0.10)	0.62	3.21 (0.01)	0.60	14.9 (0.08)	<0.001
ε3/ε3	872	2.79 (0.05)	-	3.23 (0.01)	-	16.1 (0.04)	-
ε3/ε4	357	2.72 (0.05)	0.31	3.27 (0.01)	<0.001	15.1 (0.06)	<0.001
ε2/ε4	46	3.00 (0.35)	0.33	3.21 (0.03)	0.67	13.7 (0.21)	<0.001
ε4/ε4	27	3.09 (0.17)	0.25	3.23 (0.03)	0.98	12.5 (0.16)	<0.001
ε2 carriers	188	2.84 (0.09)	0.64	3.24 (0.01)	0.59	15.2 (0.08)	<0.001
ε4 carriers	384	2.75 (0.05)	0.51	3.27 (0.01)	<0.001	14.9 (0.06)	<0.001
Phenprocoumon	220						
ε2/ε2	2	5.15 (0.65)	0.03	3.53 (0.05)	0.04	15.9 (0.35)	<0.001
ε2/ε3	33	3.20 (0.20)	0.42	3.41 (0.02)	0.62	13.6 (0.09)	<0.001
ε3/ε3	113	2.98 (0.13)	-	3.40 (0.01)	-	14.2 (0.09)	-
ε3/ε4	60	3.04 (0.18)	0.79	3.39 (0.02)	0.84	13.5 (0.10)	<0.001
ε2/ε4	4	2.53 (0.42)	0.52	3.21 (0.04)	<0.001	11.8 (0.26)	<0.001
ε4/ε4	8	2.99 (0.34)	0.99	3.55 (0.09)	0.12	21.3 (0.61)	<0.001
ε2 carriers	35	3.31 (0.20)	0.21	3.42 (0.19)	0.29	13.9 (0.09)	0.009
ε4 carriers	68	3.03 (0.16)	0.81	3.40 (0.02)	0.92	13.8 (0.10)	0.003

* Standard error of the mean.

† P-value for the difference in mean first INR after a standard start dose between the different ApoE genotypes and genotype ε3/ε3.

‡ P-value for the difference in mean INR between the different ApoE genotypes and genotype ε3/ε3. The dependency between the observations within a patient was not taken into account.

§ P-value for the difference in mean maintenance dose between the different ApoE genotypes and genotype ε3/ε3. The dependency between the observations within a patient was not taken into account.

allele frequencies from Hardy-Weinberg equilibrium. Regarding the CYP2C9 genotype, there were 1021 patients (62.4%) with the wild type genotype (CYP2C9*1/*1 homozygotes), and 552 (33.7%) with a variant genotype (1 or 2 of the mutant alleles CYP2C9*2 or CYP2C9*3). These allele and genotype proportions were also in Hardy-Weinberg equilibrium.

In Table 2 the mean first INR after a standard coumarin-starting dose, the mean INR and the mean maintenance dose are shown per ApoE genotype. For acenocoumarol-treated patients with genotypes ε2/ε2, ε2/ε3, ε3/ε4, ε2/ε4 and ε4/ε4, the mean first INR was not statistically significantly different compared to genotype ε3/ε3. The differences in mean first INR between ε2 or ε4 carriers and the reference genotype were also not statistically significant. Patients with genotype ε2/ε2 on phenprocoumon had a statistically significantly higher first INR after a standard start dose. For acenocoumarol-treated patients, the mean INR during the maintenance phase was statistically significantly higher in patients with genotype ε2/ε2 and ε3/ε4, and in ε4 carriers compared to ApoE genotype ε3/ε3. For phenprocoumon-treated patients, the mean INR during the maintenance phase was statistically sigificantly higher in patients with genotype ε2/ε2 and statistically significantly lower in patients with genotype ε2/ε4 compared

Table 3. Difference in maintenance dose for the various ApoE genotypes compared to ApoE genotype ε3/ε3

Genotype	N pat	Difference in dose compared to ε3/ε3*† (mg/week) (SEM‡)	p-value§	(95%CI)**
Acenocoumarol	1490			
ε2/ε2	14	3.5 (1.8)	0.04	(0.1)-(6.9)
ε2/ε3	174	-0.8 (0.6)	0.14	(-1.9)-(0.3)
ε3/ε3	872	-	-	-
ε3/ε4	357	-0.8 (0.4)	0.07	(-1.6)-(0.1)
ε2/ε4	46	-1.3 (1.0)	0.18	(-3.3)-(0.6)
ε4/ε4	27	-3.4 (1.3)	0.01	(-6.0)-(-0.9)
ε2 carriers	188	-0.5 (0.5)	0.37	(-1.5)-(0.6)
ε4 carriers	384	-1.0 (0.4)	0.02	(-1.8)-(-0.2)
Phenprocoumon	220			
ε2/ε2	2	1.1 (4.8)	0.82	(-8.5)-(10.7)
ε2/ε3	33	-0.4 (1.3)	0.75	(-3.1)-(2.2)
ε3/ε3	113	-	-	-
ε3/ε4	60	-0.2 (1.1)	0.85	(-2.4)-(2.0)
ε2/ε4	4	-3.2 (3.5)	0.37	(-10.1)-(3.8)
ε4/ε4	8	0.8 (2.9)	0.78	(-4.9)-(6.5)
ε2 carriers	35	-0.3 (1.3)	0.79	(-2.9)-(2.2)
ε4 carriers	68	-0.1 (1.1)	0.92	(-2.2)-(2.0)

* The mean acenocoumarol maintenance dose for genotype ε3/ε3 was 16.1 mg per week.
The mean phenprocoumon maintenance dose for genotype ε3/ε3 was 14.2 mg per week.
† Adjusted for age, gender, target INR level, measured INR, CYP2C9 genotype, and use of statins.
‡ Standard error of the mean.
§ P-value for the difference in adjusted maintenance dose between the different ApoE genotypes and genotype ε3/ε3.
** 95% confidence interval for the difference in adjusted maintenance dose between the different ApoE genotypes and genotype ε3/ε3.

to ApoE genotype ε3/ε3. In these calculations however, all INR-measurements were considered as independent observations. For patients on acenocoumarol, the mean maintenance dose was statistically significantly lower in all ApoE genotypes compared to genotype ε3/ε3, except for those with genotype ε2/ε2. These patients had a significantly higher maintenance dose. For phenprocoumon-treated patients, those with genotypes ε2/ε2 and ε4/ε4 had a significant higher maintenance dose; all other genotypes had a significant lower maintenance dose compared to the reference genotype. The dependency between the observations was also here not taken into account.

Table 3 summarizes the main findings of the study. For acenocoumarol-treated patients, ε2 homozygotes had a significantly higher maintenance dose, while patients homozygous for the ε4 allele had a statistically significantly lower maintenance dose than patients with genotype ε3/ε3. Individuals with the heterozygous genotypes had maintenance doses that were intermediate to those with the respective homozygous genotypes. These doses were not significantly different from the maintenance dose in patients with genotype ε3/ε3. The maintenance dose for ε4 carriers was also statistically significantly lower, while the dose for ε2 carriers was not significantly different from those with genotype ε3/ε3. The ε4 allele had a dose-dependent effect on the acenocoumarol maintenance dose, with a stepwise decrease

of 0.9 mg per week per allele (p = 0.007). The ε2 allele had no dose-dependent effect on the acenocoumarol maintenance dose (p = 0.69). For patients on phenprocoumon, however, maintenance doses were not statistically significantly different from those with genotype ε3/ε3. CYP2C9 genotype did not modify the association between the ApoE ε4 allele and the maintenance dose.

Patients with a variant genotype had a 21% higher risk of overanticoagulation during the first six weeks of anticoagulant therapy, compared to patients with genotype ε3/ε3, although this difference was not statistically significant (HR 1.21, 95%CI: 0.88-1.67). The total number of bleeding episodes was 241, or 9.0 per 100 treatment-years. Patients with a variant allele had a slightly but non-significantly higher risk for bleeding events (HR 1.12, 95%CI: 0.86-1.44).

DISCUSSION

The main finding in this cohort study is that individuals homozygous and heterozygous for the ε4 allele required a significantly lower dose of acenocoumarol, to reach the same level of anticoagulation, than patients with genotype ε3/ε3. Patients homozygous for the ε2 allele on acenocoumarol required a statistically significantly higher dose than patients with genotype ε3/ε3. The effect of the ε4 allele on the acenocoumarol maintenance dose was dose-dependent. Our results are in line with a previously reported small study of 30 patients on coumarins in which eight patients with the ApoE variant E4 (genotypes ε3/ε4 and ε4/ε4) had a significantly lower prothrombin content than the other 22 patients, but had used similar doses of phenprocoumon [18]. No adjustments were, however, made for potentially confounding factors. Unlike this study we found only an association between the ε4 allele and the maintenance dose in users of acenocoumarol, but not in users of phenprocoumon. This is possibly due to the relatively small number of subjects for some genotypes in phenprocoumon-treated patients in our study. In a previous study on CYP2C9 genotype and coumarin maintenance doses [19], differences in mean acenocoumarol dose compared to the wild type genotype (CYP2C9*1/*1) were -2.3, -3.5, -5.0 and -7.2 mg/week, respectively for genotypes CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2 and CYP2C9*2/*3. So, the largest reduction in mean acenocoumarol dose found in the current study for ApoE genotype ε4/ε4, is comparable with the reduction in acenocoumarol dose found for genotype CYP2C9*1/*3. For patients on phenprocoumon, no significant differences were found between variant genotypes and the wild type genotype, comparable with the current study.

These data suggest that ApoE genotype has not only a profound effect on vitamin K status [6,10,11] but also on the response to coumarin anticoagulants. It is known that patients carrying a ε4 allele have an accelerated clearance of chylomicrons from the blood [9]. It has been hypothesized that after the accelerated uptake of the vitamin K-rich chylomicron remnants in the liver, vitamin K may be less abundantly available to hepatocytes for the synthesis of

biologically active coagulation factors because of a more extensive metabolism and excretion [6,18]. This implicates, that the ApoE ε4 allele not only enhances ApoE-receptor mediated hepatic extraction of chylomicron remnants from plasma, but also affects the intra-hepatocyte routing of extracted compounds, i.e. the entered vitamin K from chylomicrons is directly eliminated and bypasses the intra-cellular vitamin K dependent biochemical system. As a consequence, such patients would require a lower dose of coumarins, which competitively inhibit vitamin K, and might react with a stronger anticoagulation reponse upon therapeutic challenge with a standard dose of coumarin anticoagulants. However, the clinical consequences of carrying a variant allele seem to be mild. Patients with a variant genotype have a slightly increased INR but the risk of bleeding events seems to be negligible. It seems likely that even in patients with the ε4/ε4 genotype, regular monitoring of the INR strongly reduces the risk of bleeding. This is in contrast with a previous study on CYP2C9 and bleeding risk [20], where we found that carrying a variant allele of CYP2C9 was associated with an increased risk of major bleeding events in patients on acenocoumarol.

Most studies on ApoE and vitamin K, including our own, have focussed on the same polymorphism in the coding region of the apolipoprotein E gene. This coding polymorphism defines the ApoE isoforms E2, E3 and E4. However, in addition to this common genetic polymorphism, several other variations in the ApoE gene have been reported [21]. Of interest are polymorphisms identified in the promotor region. Their major impact is to affect the ApoE gene expression, and consequently, ApoE isoform concentrations [21-24]. Whether or not differences in absolute levels of ApoE are associated with coumarin anticoagulant doses deserves further investigation. The allelic frequencies found in our population are within the range of reported allele frequencies among other Caucasian populations. In Europeans and American Caucasians the relative frequency of the ε4 allele ranges from 0.10 to 0.23 [25-28].

Some potential limitations of our cohort study should be considered. Selection bias was probably negligible because we identified all users of coumarin anticoagulants in a defined population and difficulties with genotyping were random. Furthermore, our study population was in Hardy-Weinberg equilibrium, suggesting that no selection has occurred among genotypes, which could have explained the observed association. Also, information bias is not likely as all data on genotype and outcome were gathered prospectively and recorded similarly for all cohort members without prior knowledge of our study hypothesis. Potential confounding by age, gender, target INR level, measured INR, CYP2C9 genotype, and use of statins was dealt with in the multivariate analyses, although it seems largely redundant to adjust for additional confounding factors, because a confounding factor should be associated with ApoE genotype in order to distort the studied association.

In conclusion, individuals homozygous or heterozygous for the ε4 allele required a significantly lower dose of acenocoumarol, to reach the same level of anticoagulation, than patients with genotype ε3/ε3. Acenocoumarol-treated patients homozygous for the ε2 allele

required a significantly higher dose than those with genotype ε3/ε3. As all coumarins are competitors of vitamin K, it is likely that our results also apply to warfarin.

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Chapter 3

Drug interactions with coumarin anticoagulants

Chapter 3.1

Overanticoagulation associated with combined use of antibacterial drugs and acenocoumarol or phenprocoumon anticoagulants

ABSTRACT

Introduction: Several case reports associated combined use of coumarins and antibacterial drugs with overanticoagulation. Despite the fact that these drugs are frequently prescribed concurrently, there is little quantitative information on the risks of such complications.

Objective: To study which antibacterial drugs are associated with overanticoagulation during therapy with coumarins.

Design: Population-based cohort study in a sample of the Rotterdam Study.

Subjects: All patients who were treated with acenocoumarol or phenprocoumon in the study period from April 1, 1991 through December 31, 1998 and for whom international normalised ratio (INR) data were available.

Methods: Patients were followed until an INR $\geq$ 6.0, the end of their treatment, death or end of the study period. Proportional hazards regression analysis was used to estimate the risk of an INR $\geq$ 6.0 in relation to concomitant use of a coumarin anticoagulant and antibacterial drugs after adjustment for several potentially confounding factors such as age, gender, hepatic dysfunction, malignancies, and heart failure.

Results: Of the 1124 patients in the cohort, 351 had an INR $\geq$ 6.0. The incidence rate was 6.9 per 10,000 treatment days. Sulfamethoxazole combined with trimethoprim most strongly increased the risk of overanticoagulation with an adjusted relative risk of 20.1 (95% confidence interval: 10.7-37.9). Stratification showed that the induction period of overanticoagulation varied between different antibacterial drugs.

Conclusion: In this study among outpatients of an anticoagulation clinic using acenocoumarol or phenprocoumon, several antibacterial drugs strongly increased the risk of overanticoagulation. Awareness of these drug interactions and more frequent monitoring of INR values during the initial stages of antibacterial drug therapy are warranted to minimize the risk of bleeding complications.

INTRODUCTION

Coumarin anticoagulants are extensively used for the treatment and long-term prevention of thromboembolic diseases [1, 2]. These drugs induce their anticoagulant effect by antagonising vitamin K, thereby impairing the biological activity of the vitamin K-dependent coagulation factors [3, 4]. The risk of bleeding, the main complication of coumarin anticoagulants, is influenced by the intensity of anticoagulant therapy [5-9], by the patient's underlying clinical disorder [9, 10], and by the concomitant use of other drugs [2, 11, 12]. This risk sharply increases when the international normalised ratio (INR) is $\geq$ 6.0 [13, 14]. Growing experience with anticoagulant therapy, and increased understanding of drug interactions, has reduced the number of bleeding complications [15]. Several drugs can affect the prothrombin time during coumarin anticoagulant therapy by different mechanisms [11, 12, 16, 17]. During the past decades, many case reports associated the use of antibacterial drugs with overanticoagulation [18-24]. Prospective studies investigated this association, but these were conducted in young healthy volunteers, were only able to identify interactions that occur relatively frequently and were mostly limited to warfarin [25-31]. A recent case-control study suggested that antibacterial drugs are an important cause of overanticoagulation [32]. In that study, however, data on drug use came from patient interview and the sample size was too small to study the effect of different antibacterial agents on coumarins. Therefore, we conducted a follow-up study in a large population-based cohort to investigate which antibacterial drugs are associated with overanticoagulation during therapy with coumarins.

METHODS

Setting

Data were obtained from the Rotterdam Study and from the regional outpatient anticoagulation clinic. The Rotterdam Study is a prospective population-based cohort study of neurological, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, aged 55 years or over were invited in 1990-1993 to participate in the study. The rationale and design of this study have been described elsewhere [33]. The cohort encompasses 7983 individuals who were all interviewed and investigated at baseline. During baseline interview, data were gathered on medical history, and a food questionnaire was completed. During a subsequent visit to the research center, patients were examined and blood was taken for the assessment of electrolytes, liver enzyme levels, serum creatinine, serum albumin and several other laboratory values. The anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. Prothrombin times are monitored each one to six weeks by reference to the INR, dependent on the stability of the anticoagulant level.

Doses are adjusted on the basis of the INR of the patient by computerised dose calculations. For this study data were used from January 1, 1991 through December 31, 1998. More than 99% of participants fill their drugs at seven regional pharmacies, which are fully computerised. Complete data on drug use were available as of January 1, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code [34], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs.

Cohort and outcome definition

The study cohort consisted of all 1124 patients in the Rotterdam Study, who were treated with acenocoumarol or phenprocoumon in the study period between April 1, 1991 and December 31, 1998 and for whom there were INR data from the anticoagulation clinic during their treatment. The start date April 1 was chosen to ensure that at least 3 months of medication history from the pharmacy was available for every cohort member. The cohort included prevalent users of coumarins on the starting date and incident users during the study period. All cohort members were followed as of April 1, 1991 for prevalent users and from their first INR assessment for incident users. Both groups were followed until the first occurrence of an INR $\geq$ 6.0, the last INR assessment because of the end of their treatment, death or end of the study period, whichever came first. This means that during follow-up, all study members were anticoagulated and regularly assessed for their INR. The date on which an INR $\geq$ 6.0 was encountered was defined as the index date.

Cofactors

The following baseline patient characteristics were considered as potential determinants for affecting the response of the INR to coumarin anticoagulants: gender, age, hepatic dysfunction (defined as serum aminotransferases > 2x the upper level of normal), hypoalbuminemia ($\leq$ 35 g/l), malignancies, hyperthyroidism, hypertension (systolic blood pressure $\geq$ 160 mm Hg and/or diastolic blood pressure $\geq$ 95 mm Hg or use of antihypertensives), heart failure, and low dietary intake of vitamin K (< 1 µg/kg/day). In addition, we considered duration of follow-up and whether the INR measurement on the index date was earlier than according to the INR monitoring scheme. Furthermore, to study the potentially confounding effect of fever or of the indication for treatment we studied the presence or absence of these features in the medical records of the general practitioners. We did this validation for all cases and a random sample from the remainder of the cohort who all had been treated with antibacterial drugs on the index date.

Statistical analysis

Incidence rates were calculated by dividing the number of cases of an INR $\geq$ 6.0 by the number of days on combined use of a coumarin anticoagulant and an antibacterial drug. To assess antibacterial drugs which were independently associated with an INR $\geq$ 6.0, all occurring

combinations of a coumarin anticoagulant and an antibacterial drug were included separately in a Cox proportional hazards regression model for time-dependent variables to compute relative risks and their 95% confidence intervals (95%CI) [35]. The model compares exposure to this combination on the index date of each case with that of all other non-censored subjects in the cohort on the same date as the case. To adjust for potential confounding, cofactors which were associated with an INR ≥ 6.0 in the univariate analysis were included in the multivariate model if this caused a change in the point estimate of more than 5 percent. In order to study the time interval between the first treatment day with an antibacterial drug and an INR value ≥ 6.0, we distinguished 2 intervals: 1-3 days and ≥ 4 days. Stratification by these time-intervals was performed because the induction period differs per mechanism by which antibacterial drugs may cause overanticoagulation. If possible, stratified analyses by the type of anticoagulant were performed. Moreover, separate analyses were performed for prevalent and incident users of coumarin anticoagulants on the starting date.

RESULTS

Of the 1124 patients in the cohort, 351 developed an INR ≥ 6.0 after April 1, 1991. The incidence rate was 6.9 per 10,000 treatment days. Baseline characteristics of cases and the total cohort

Table 1. Characteristics of patients with an INR ≥ 6.0 and the total cohort

	Patients with INR ≥ 6.0 n = 351	Total cohort n = 1124	RR*	(95%CI)
Gender				
Male	177 (50.4%)	534 (47.5%)	1.00	Reference
Female	174 (49.6%)	590 (52.5%)	1.44	(1.17-1.77)
Age (years, mean (SD))	73 (8)	72 (8)	1.04	(1.03-1.05)
55-64	49 (14.0%)	218 (19.4%)	1.00	Reference
65-74	152 (43.3%)	496 (44.1%)	1.28	(0.92-1.76)
75-84	124 (35.3%)	340 (30.2%)	1.85	(1.33-2.58)
≥ 85	26 (7.4%)	70 (6.2%)	3.17	(1.96-5.14)
Type of anticoagulant				
Acenocoumarol	295 (84.0%)	951 (84.6%)	1.00	Reference
Phenprocoumon	56 (16.0%)	173 (15.4%)	0.57	(0.43-0.76)
Hepatic dysfunction	3 (0.9%)	12 (1.1%)	3.75	(1.18-11.9)
Hypoalbuminemia	1 (0.3%)	2 (0.2%)	1.16	(0.16-8.30)
Malignancies	43 (12.3%)	94 (8.4%)	1.67	(1.21-2.30)
Hyperthyroidism	12 (3.4%)	37 (3.3%)	1.51	(0.85-2.70)
Hypertension	148 (42.2%)	434 (38.6%)	1.08	(0.86-1.35)
Heart failure	83 (23.7%)	141 (12.5%)	1.63	(1.27-2.09)
Low intake of vitamin K	7 (2.0%)	11 (1.0%)	3.74	(1.96-7.95)

* Univariate analyses of relative risks were performed with a Cox proportional-hazards model. Relative risks cannot be calculated with the numbers in this table because controls may later become cases.

Table 2. Association between overanticoagulation (INR ≥ 6.0) and use of antibacterial drugs

Antibacterial drug	Cases	Cohort*	IR†	RR_{crude} (95%CI)‡	$RR_{adj.}$ (95%CI)§
	n = 351 (No.)	n = 1124 (No.)			
Amoxicillin	8	180	32.0	11.1 (5.4-22.9)	10.5 (5.1-21.7)
Amoxicillin & enzyme inhitor	4	205	18.0	4.6 (1.7-12.4)	5.1 (1.9-13.9)
Amphotericin	0	11		P = 0.89	
Azithromycin	0	2		P = 0.95	
Cefalexin	0	3		P = 0.94	
Cefixime	0	2		P = 0.95	
Cefuroxime	0	4		P = 0.93	
Ciprofloxacin	0	19		P = 0.85	
Clarithromycin	3	69	78.7	11.0 (3.4-35.4)	11.7 (3.6-37.8)
Clindamycin	0	37		P = 0.80	
Doxycycline	5	288	32.3	4.2 (1.7-10.2)	4.3 (1.8-10.4)
Erythromycin	0	78		P = 0.69	
Flucloxacillin	0	25		P = 0.83	
Norfloxacin	3	66	44.6	10.0 (3.1-32.1)	9.8 (3.0-31.6)
Ofloxacin	0	33		P = 0.81	
Pheniticillin	1	219	0.2	1.2 (0.2-8.3)	0.9 (0.1-6.1)
Roxithromycin	0	14		P = 0.86	
Sulfamethoxazole & trimethoprim	11	125	154.7	20.1 (10.7-37.7)	20.1 (10.7-37.9)
Trimethoprim	2	61	26.8	6.0 (1.4-25.2)	5.6 (1.3-23.1)
Vancomycin	1	10	15.9	17.7 (2.3-138.0)	13.6 (1.7-106.8)

* In this time-dependent analysis, exposure in cases and controls is assessed on every index date. As a consequence, the number of assessments in the reference group is much larger than the number of individuals. Hence, crude relative risks cannot be calculated with the numbers in Table 2.
† Expressed as the number of cases per 10,000 days on combined use of a coumarin and an antibacterial drug.
‡ If none of the cases was exposed, p-values are given instead of relative risks.
§ Adjusted for gender and age.

are shown in Table 1. Women and older patients had a higher risk of an INR ≥ 6.0. The risk of overanticoagulation was lowest with phenprocoumon. Hepatic dysfunction, malignancies, heart failure and a low dietary daily intake of vitamin K were associated with an increased risk of an INR ≥ 6.0 in the univariate analysis. There was no difference in duration of follow-up between cases and the total cohort (mean 559±481 days). The INR measurement on the index date was earlier than planned in 8.6% of the cases and in 5.6% of the total cohort (p = 0.0048).

Twenty different antibacterial drugs were used during the study period, of which eleven were not used in cases. Thirty-eight cases (11%) used antibacterial drugs on the index date. Incidence rates for the individual antibacterial drugs are presented in Table 2. Eight antibacterial drugs were univariately as well as multivariately associated with overanticoagulation. The relative risk varied considerably between the different drugs (Table 2). Sulfamethoxazole combined with trimethoprim (co-trimoxazole) most strongly increased the risk of overanticoagulation. Only age and gender were associated with a change of the point estimate of more than five percent. After adjustment for these confounding factors the relative risk was 20.1 (95%CI: 10.7-37.9). There was no significant difference in the frequency of fever in cases on antibacterial drugs

Table 3. Association between overanticoagulation (INR ≥ 6.0) and use of antibacterial drugs stratified by time interval*

Antibacterial drug	$RR_{adj.}$ (95%CI)† overall	$RR_{adj.}$ (95%CI)† 1-3 days	$RR_{adj.}$ (95%CI)† ≥ 4 days
Amoxicillin	10.5 (5.1-21.7)	7.2 (1.8-29.7)	13.2 (5.6-30.8)
Amoxicillin & enzyme inhibitor	5.1 (1.9-13.9)	-	7.3 (2.7-19.9)
Clarithromycin	11.7 (3.6-37.8)	21.3 (5.0-90.6)	6.3 (0.9-46.3)
Doxycycline	4.3 (1.8-10.4)	2.9 (0.4-20.9)	5.2 (1.9-14.0)
Norfloxacin	9.8 (3.0-31.6)	19.3 (4.5-83.7)	5.0 (0.7-36.6)
Pheniticillin	0.9 (0.1-6.1)	-	0.9 (0.1-6.5)
Sulfamethoxazole & trimethoprim	20.1 (10.7-37.9)	16.6 (5.1-54.4)	23.2 (10.9-49.1)
Trimethoprim	5.6 (1.3-23.1)	9.0 (1.2-67.4)	4.1 (0.6-30.6)
Vancomycin	13.6 (1.7-106.8)	-	15.1 (1.9-120.0)

* Time interval is the interval between the first treatment day with an antibacterial drug and an INR ≥ 6.0.
† Adjusted for gender and age.

and individuals in the rest of the cohort who were treated with antibacterial drugs on the index date. Similarly, there was no difference in the indication for antibacterial drugs between cases and the remainder of the cohort.

Stratification by time-interval revealed that the adjusted relative risks of overanticoagulation by antibacterial drugs were different for both time intervals (Table 3). Relative risks could not be established for both time periods for all antibacterial drugs because overanticoagulation only occurred in one of the time intervals for some drugs. For amoxicillin and sulfamethoxazole-trimethoprim, relative risks were significantly increased for both time intervals. When comparing the relative risks for both time intervals, the antibacterial drugs with the highest relative risk 1-3 days after start of use were clarithromycin, norfloxacin and trimethoprim. For amoxicillin, doxycycline, sulfamethoxazole-trimethoprim relative risks of overanticoagulation were most strongly increased ≥ 4 days after start of the antibacterial drug.

Stratified analyses by the type of anticoagulant could only be performed for amoxicillin and sulfamethoxazole with trimethoprim. For patients on acenocoumarol the adjusted relative risk of amoxicillin was 8.0 (95%CI: 3.5-18.4). For users of phenprocoumon the adjusted relative risk of amoxicillin was 22.4 (95%CI: 4.0-126.3). Sulfamethoxazole with trimethoprim was associated with an adjusted relative risk of overanticoagulation of 17.3 (95%CI: 8.6-34.9) in patients on acenocoumarol, and 14.3 (95%CI: 2.8-73.8) in patients on phenprocoumon. Numbers appeared to be too small to calculate risks in prevalent users on the starting date. In incident users risks were largely the same as in the total population (data not shown).

DISCUSSION

The main finding in this population-based cohort study is that several antibacterial drugs were associated with a strongly increased risk of overanticoagulation during coumarin anticoagulant

therapy with acenocoumarol or phenprocoumon. It is likely that these results can be extrapolated to warfarin. Relative risks varied considerably between the different antibacterial drugs, especially after stratification for the time-interval between start of the antibacterial drug therapy and the moment of overanticoagulation. The strongest risk increase was associated with sulfamethoxazole-trimethoprim. This is in line with an earlier study [32].

Overanticoagulation may be caused by pharmacokinetic or pharmacodynamic interactions between antibacterial drugs and coumarins. Pharmacokinetic interactions may be caused by plasma protein binding displacement of coumarins or by inhibition of the metabolism of coumarins. Plasma protein binding displacement is a rapid and short-lived effect because the displaced molecules are rapidly metabolised. A small but transient increase in anticoagulant effect can occur [16]. Usually this mechanism plays a minor role compared to other mechanisms [16]. It may be clinically relevant in the elderly, however, in whom plasma protein binding decreases [36]. Unlike enzyme induction, which may take several days or even weeks to develop, inhibition of cytochrome P450 enzymes can occur within two to three days, depending on the elimination half-life of the inhibited drug [16]. Pharmacodynamic interactions may result from vitamin K deficiency by elimination of bacterial flora in the gut, and by direct inhibition of the synthesis of the vitamin K dependent coagulation factors [2, 16]. Pharmacodynamic mechanisms take at least several days to develop because of already circulating coagulation factors [16]. It has been stated that the contribution of bacterial synthesis to vitamin K status in man becomes important only when the dietary intake of the vitamin is markedly decreased [12, 37]. Unfortunately, we were not able to investigate this as there were no case patients in our study with a low daily intake of vitamin K who had used antibacterial drugs. Although acenocoumarol and phenprocoumon have a similar effect on the elimination of bacterial flora in the gut and the direct inhibition of the synthesis of vitamin K dependent coagulation factors, the pharmacokinetic properties differ distinctly. Especially the variability in elimination kinetics, could cause a difference in the contribution of the metabolic component. For amoxicillin, relative risks were significantly increased during both time intervals, suggesting that more than one mechanism may be involved. In the literature, nothing was found about possible pharmacokinetic interactions of coumarins with amoxicillin. Hence, the increased risk during the first days after start of amoxicillin is surprising. When amoxicillin was combined with an enzyme inhibitor, however, the relative risk was much lower. For clarithromycin, the relative risk of overanticoagulation was only significantly increased during the first 3 days after start of use. This is in accordance with the literature in which the most frequently suggested mechanism is the inhibition of the hepatic cytochrome P450 mixed-function oxidase system, resulting in a reduced clearance of coumarin anticoagulants and an increase in its effect [16, 18]. For doxycycline, several mechanisms have been suggested, but in all published case reports overanticoagulation developed within 7 to 10 days [23, 24]. Our data confirm this. For norfloxacin it is clear from our results that the rapid pharmacokinetic interactions are far more important than the delayed pharmacodynamic ones. Mechanisms suggested for norfloxacin to increase

prothrombin times are plasma protein binding displacement [16] and inhibition of metabolic clearance [38]. For sulfamethoxazole-trimethoprim the relative risk of overanticoagulation was strongly increased during the first 3 days of antibacterial drug therapy as well as thereafter. Sulfonamides can strongly displace coumarin anticoagulants from their plasma protein binding sites [16] and it has been suggested that sulfamethoxazole-trimethoprim, which is metabolised by the CYP2C9 isoenzyme, increases the anticoagulant effect by inhibiting the metabolism of the anticoagulant [39]. The degree of inhibition of the metabolism may be different for acenocoumarol and phenprocoumon [40] as acenocoumarol (like warfarin) is predominantly metabolised by CYP2C9 [41]. None of the other antibacterial drugs examined is metabolised by CYP2C9. Especially after $\geq$ 4 days, sulfamethoxazole is probably largely responsible for the increased prothrombin times considering the lower relative risk we found for trimethoprim alone. Vancomycin will probably, as a poorly absorbed drug, mainly reduce the bacterial synthesis of vitamin K and thereby increase the INR with a delayed onset [16]. This is compatible with our results. However, for vancomycin, as well as for trimethoprim, numbers were too small to draw firm conclusions.

Some potential limitations should be considered in the interpretation of our cohort study. Selection bias was probably negligible as we identified all users of coumarin anticoagulants in a defined population and because regular INR monitoring makes it unlikely that cases were missed. Also, information bias is not likely as all data on exposure and outcome were recorded similarly for subjects exposed and not exposed to combinations of antibacterial drugs and coumarin anticoagulants without prior knowledge of our study hypothesis. Potential confounding by gender, age, hepatic dysfunction, hypoalbuminemia, malignancies, hyperthyroidism, hypertension, heart failure, low dietary intake of vitamin K, differences in duration of follow-up and INR measurement as scheduled, was dealt with in the multivariate analyses. Whether the INR assessment on the index date was earlier than according to plan was taken into consideration because patients who are prescribed a known potentially hazardous combination are more likely to be monitored with short intervals. However, neither adjustment for this nor for the average number of assessments during follow-up changed our results. Fever may be a confounding factor as a hypermetabolic state produced by fever might potentiate the response to coumarin anticoagulants, probably by increasing the catabolism of vitamin K-dependent coagulation factors [2]. There was, however, no significant difference between the frequency of fever in cases on antibacterial drugs and individuals in the rest of the cohort who were treated with antibacterial drugs on the index date. Furthermore, we excluded confounding by indication, as there was no significant difference in the indication for antibacterial agents between cases and the remainder of the cohort. Moreover, confounding by indication would not explain the variation in relative risks between antibacterial agents and would occur similarly for all antibacterial drugs.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic using acenocoumarol or phenprocoumon, several antibacterial drugs

were strongly associated with overanticoagulation. The onset of overanticoagulation after start of antibacterial drug therapy varied between drugs. The risk was most strongly increased by a course of amoxicillin, clarithromycin, norfloxacin and sulfamethoxazole-trimethoprim. Awareness of these drug interactions and more frequent monitoring during the initial stages of antibacterial drug therapy to maintain patients on coumarins such as warfarin, acenocoumarol and phenprocoumon within the recommended therapeutic ranges may minimize the risk of bleeding complications.

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Chapter 3.2

Overanticoagulation associated with combined use of antifungal agents and coumarin anticoagulants

ABSTRACT

Introduction: Several case reports have associated combined use of coumarins and antifungal agents with overanticoagulation. However, we are not aware of epidemiological studies that quantify the risk of overanticoagulation caused by antifungal agents. We conducted a follow-up study in a large population-based cohort to investigate the antifungal agents that are associated with overanticoagulation during therapy with coumarins.

Methods: All patients in the Rotterdam Study who were treated with acenocoumarol or phenprocoumon in the study period from April 1, 1991 through December 31, 1998 and for whom international normalised ratio (INR) data were available, were followed until an INR $\geq$ 6.0, the end of their treatment, death or end of the study period. Proportional hazards regression analysis was used to estimate the risk of an INR $\geq$ 6.0 in relation to concomitant use of a coumarin anticoagulant and antifungal agents after adjustment for several potentially confounding factors such as age, gender, hepatic dysfunction, malignancies, and heart failure.

Results: Of the 1124 patients in the cohort, 351 had an INR $\geq$ 6.0. The incidence rate was 6.9 per 10,000 treatment days. Oral miconazole most strongly increased the risk of overanticoagulation with an adjusted relative risk of 36.6 (95% confidence interval: 12.4-108.0).

Conclusions: In this study among outpatients of an anticoagulant clinic on coumarins, oral miconazole was especially strongly associated with overanticoagulation. Awareness of these drug interactions and more frequent monitoring of INR values during the initial stages of treatment with some antifungal drugs in patients on coumarins may minimise the risk of bleeding complications. The concurrent use of miconazole and coumarins should be discouraged.

INTRODUCTION

Coumarin anticoagulants are used extensively for the treatment and long-term prevention of thromboembolic diseases [1, 2]. These drugs induce their anticoagulant effect by antagonising vitamin K, thereby impairing the biological activity of the vitamin K-dependent coagulation factors [3, 4]. The risk of bleeding, the main complication of coumarin anticoagulants, is influenced by the intensity of anticoagulant therapy [5-9], by the underlying clinical disorders of the patient [9, 10], and by the concomitant use of other drugs [2, 11, 12]. This risk sharply increases when the international normalised ratio (INR) is $\geq$ 6.0 [13, 14]. Growing experience with anticoagulant therapy, and increased understanding of drug interactions has reduced the number of bleeding complications [15]. Several drugs can affect the INR during coumarin anticoagulant therapy by different mechanisms [11, 12, 16, 17]. During the past decades, many case reports and small-scale experiments associated the use of antifungal agents with overanticoagulation [18-25]. Several reviews discussed the clinical evidence for and importance of interactions involving antifungal agents [26-31]. So far as we are aware, however, epidemiological studies quantifying the risk of overanticoagulation by antifungal agents in a nonselected population on coumarins have not been done before. Therefore we conducted a follow-up study in a large population-based cohort to investigate which antifungal agents are associated with overanticoagulation during therapy with coumarins.

METHODS

Setting

Data were obtained from the Rotterdam Study and from the regional outpatient anticoagulation clinic. The Rotterdam Study is a prospective population-based cohort study of neurologic, cardiovascular, locomotor and ophthalmologic diseases. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, aged 55 years or more were invited in 1990-1993 to participate in the study. The rationale and design of this study have been described elsewhere [32]. The cohort encompasses 7983 individuals who were all interviewed and investigated at baseline. The anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The type of anticoagulant is chosen by the physician. INRs are monitored every one to six weeks, dependent on the stability of the INR. Doses are adjusted on the basis of the INR of the patient by computerised dose calculations. More than 99% of participants have their drug prescriptions filled at seven regional pharmacies, which are fully computerised. Complete data on drug use were available as of January 1, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code [33], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs.

Cohort and outcome definition

The study cohort consisted of all 1124 patients in the Rotterdam Study, who were treated with acenocoumarol or phenprocoumon in the study period between April 1, 1991 and December 31, 1998 and for whom there were INR data from the anticoagulation clinic during their treatment. The start date of April 1, 1991 was chosen to ensure that at least 3 months of medication history from the pharmacy was available for every cohort member. The cohort included prevalent users of coumarins on the starting date and incident users during the study period. All cohort members were followed as of April 1, 1991 for prevalent users and from their first INR assessment for incident users. Both groups were followed until the first occurrence of an INR $\geq$ 6.0, the last INR assessment because of the end of their treatment, death or end of the study period, whichever came first. This means that during follow-up all study members underwent anticoagulation and were regularly assessed for the INR. The date on which an INR $\geq$ 6.0 was encountered was defined as the index date.

Exposure definition

We assessed exposure to the following antifungal agents on the index date: the imidazoles - bifonazole, butaconazole, clotrimazole, econazole, ketoconazole, miconazole, and sulconazole; the triazoles - fluconazole, itraconazole, and terconazole; the polyene macrolides - amphotericin and nystatin; the allylamine terbinafine; flucytosine; and griseofulvin. The different routes of administration were studied separately.

Cofactors

The following baseline patient characteristics were considered as potential determinants for affecting the response of the INR to coumarin anticoagulants: gender, age, hepatic dysfunction (defined as serum aminotransferases of greater than 2 times the upper level of normal), hypoalbuminemia ($\leq$ 35 g/l), malignancies, hyperthyroidism, hypertension (either a systolic blood pressure $\geq$ 160 mm Hg or a diastolic blood pressure $\geq$ 95 mm Hg, or both, or use of antihypertensives), and heart failure. In addition, we considered duration of follow-up and whether the INR measurement on the index date was earlier than expected according to the INR monitoring scheme. We considered all agents as potentially confounding drugs that have have been associated with overanticoagulation in the medical literature or discontinuation of known inducers of cytochrome P450 (CYP) enzymes within the week before the index date [17, 34]. The cessation of drugs that induce the microsomal oxidase system can down-regulate the metabolism of coumarins in patients who are stably anticoagulated. This may lead to increased levels of circulating anticoagulant and thereby cause overanticoagulation. Furthermore, to study the potentially confounding effect of fever or indication for treatment, we validated by reference to the medical records of the general practitioner all cases and a random sample from the remainder of the cohort who had been treated with oral antifungal agents on the index date.

Statistical analysis

Incidence rates were calculated by dividing the number of cases of an INR ≥ 6.0 by the number of days on combined use of a coumarin anticoagulant and an antifungal agent. To assess antifungal agents that were independently associated with an INR ≥ 6.0, all occurring combinations of a coumarin anticoagulant and an antifungal agent were included separately in a Cox proportional hazards regression model for time-dependent variables to compute relative risks and their 95% confidence intervals (95%CI) [35]. The model compares exposure to this combination at the index date of each case with that of all subjects in the cohort who are at risk for the outcome of interest. To adjust for potential confounding, cofactors that were associated with an INR ≥ 6.0 in the univariate analysis were included in the multivariate model, in addition to gender and age, if this caused a change in the point estimate of more than 5 percent. If possible, separate analyses were performed for acenocoumarol and phenprocoumon.

RESULTS

Of the 1124 patients in the cohort, 351 had an INR ≥ 6.0 after April 1, 1991. The incidence rate was 6.9 per 10,000 treatment days. Baseline characteristics of patients with an INR ≥ 6.0 and the total population are shown in Table 1. Women and older patients had a higher risk of an INR ≥ 6.0. The risk of overanticoagulation was lowest with phenprocoumon. Hepatic dysfunction, malignancies and heart failure were associated with an increased risk of an INR ≥ 6.0 in the univariate analysis. There was no difference in duration of follow-up between cases and the total cohort (mean ± SD, 559 ± 481 days). The INR measurement on the index date was earlier than planned in 8.6% of the cases and in 5.6% of the total cohort (p = 0.0048).

Nine different antifungal agents in different formulations were used during the study period; four of these were not used in cases (Table 2). Patients exposed to the following antifungal applications did not have a higher risk of overanticoagulation: bifonazole (cutaneous) (p = 0.95), clotrimazole (vaginal) (p = 0.97), fluconazole (oral) (p = 0.87), amphotericin (oral) (p = 0.89), nystatin (cutaneous) (p = 0.93) and terbinafine (both oral and cutaneous) (p = 0.76 and p = 0.82, respectively). Thirteen (4%) patients used antifungal agents on the index date. Four antifungal agents were univariately as well as multivariately associated with overanticoagulation (Table 2). The relative risk varied considerably between the different agents and the different routes of administration. Oral miconazole most strongly increased the risk of overanticoagulation. After adjustment for confounding factors, the relative risk was 36.6 (95%CI: 12.4-108.0). For the vaginal and cutaneous administration of miconazole, relative risks were substantially lower: 4.3 (95%CI: 0.6-31.6) and 1.4 (95%CI: 0.4-4.3), respectively. None of the case patients receiving antifungal agents had fever on the index date or in the preceding week. Furthermore, there was no difference in the indication for antifungal agents between cases and the remainder of the cohort (mainly treatment of candidiasis, p = 0.87).

Table 1. Association between overanticoagulation (INR ≥ 6.0) and sociodemographic and comorbid conditions

	Patients with INR ≥ 6.0 n = 351	Total study Cohort n = 1124	RR*	(95%CI)
Gender				
Male	177 (50.4%)	534 (47.5%)	1.00	Reference
Female	174 (49.6%)	590 (52.5%)	1.44	(1.17-1.77)
Age (yrs, mean ± SD)	73 ± 8	72 ± 8	1.04	(1.03-1.05)
55-64	49 (14.0%)	218 (19.4%)	1.00	Reference
65-74	152 (43.3%)	496 (44.1%)	1.28	(0.92-1.76)
75-84	124 (35.3%)	340 (30.2%)	1.85	(1.33-2.58)
≥ 85	26 (7.4%)	70 (6.2%)	3.17	(1.96-5.14)
Type of anticoagulant				
Acenocoumarol	295 (84.0%)	951 (84.6%)	1.00	Reference
Phenprocoumon	56 (16.0%)	173 (15.4%)	0.57	(0.43-0.76)
Hepatic dysfunction†				
Absent	233 (66.4%)	757 (67.3%)	1.00	Reference
Present	3 (0.8%)	12 (1.1%)	3.75	(1.18-11.9)
Hypoalbuminemia†				
Absent	243 (69.2%)	781 (69.5%)	1.00	Reference
Present	1 (0.3%)	2 (0.2%)	1.16	(0.16-8.30)
Malignancies†				
Absent	308 (87.7%)	1030 (91.6%)	1.00	Reference
Present	43 (12.3%)	94 (8.4%)	1.67	(1.21-2.30)
Hyperthyroidism†				
Absent	313 (89.2%)	1013 (90.1%)	1.00	Reference
Present	12 (3.4%)	37 (3.3%)	1.51	(0.85-2.70)
Hypertension†				
Absent	161 (45.8%)	585 (52.0%)	1.00	Reference
Present	148 (42.2%)	434 (38.6%)	1.08	(0.86-1.35)
Heart failure†				
Absent	268 (76.3%)	983 (87.5%)	1.00	Reference
Present	83 (23.7%)	141 (12.5%)	1.63	(1.27-2.09)

* Univariate analyses of relative risks were performed with a Cox proportional-hazards model. Relative risks cannot be calculated with the numbers in this table because controls may later become cases.
† Totals do not add up to column totals because of missing values.

Stratified analyses by type of anticoagulant could be performed only for oral miconazole. For patients on acenocoumarol, the adjusted relative risk of oral miconazole was 35.1 (95%CI: 10.1-121.8). For users of phenprocoumon, the adjusted relative risk of oral miconazole was 16.5 (95%CI: 1.3-212.6).

Table 2. Association between overanticoagulation (INR ≥ 6.0) and use of antifungal agents

Antifungal agent	Patients with INR ≥ 6.0	Total study cohort*	IR†	RR_{crude} ‡ (95%CI)	$RR_{adj.}$ § (95%CI)
	n = 351 (No.)	n = 1124 (No.)			
Imidazoles					
Bifonazole, cutaneous	0	2	-	P = 0.95	
Clotrimazole, cutaneous	1	13	61.3	20.0 (2.6-152.7)	13.8 (1.8-108.4)
Clotrimazole, vaginal	0	1	-	P = 0.97	
Ketoconazole, cutaneous	2	500	0.3	0.9 (0.2-3.8)	1.1 (0.3-4.3)
Miconazole, oral	4	24	94.1	39.7 (13.5-116.4)	36.6 (12.4-108.0)
Miconazole, vaginal	1	44	8.7	5.5 (0.8-40.2)	4.3 (0.6-31.6)
Miconazole, cutaneous	3	589	1.4	1.2 (0.4-3.8)	1.4 (0.4-4.3)
Triazoles					
Fluconazole, oral	0	5	-	P = 0.87	
Itraconazole, oral	1	9	25.1	15.2 (1.9-120.6)	13.9 (1.7-115.0)
Polyene macrolides					
Amphotericin, oral	0	11	-	P = 0.89	
Nystatin, cutaneous	0	4	-	P = 0.93	
Nystatin, oral	1	24	9.2	11.1 (1.5-81.7)	10.3 (1.4-76.6)
Allylamines					
Terbinafine, cutaneous	0	29	-	P = 0.82	
Terbinafine, oral	0	49	-	P = 0.76	

* In this time-dependent analysis, exposure in case patients and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). Because control patients can be used multiple times, the number of assessments in the reference group is much larger than the number of individuals. Hence, crude relative risks cannot be calculated with the numbers in Table 2.
† Expressed as the number of patients with an INR ≥ 6.0 per 10,000 days on combined use of a coumarin anticoagulant and an antifungal agent.
‡ If none of the patients with an INR ≥ 6.0 was exposed, p-values are given instead of relative risks.
§ Adjusted for gender, age, malignancies and heart failure.

DISCUSSION

The main finding in this population-based cohort study is that some antifungal agents were associated with a strongly increased risk of overanticoagulation during coumarin anticoagulant therapy with acenocoumarol or phenprocoumon. The strongest risk increase was associated with oral miconazole. In many countries warfarin is the coumarin of first choice. The results of our study, however, will largely apply to these countries as well. First, the difference in half-life between coumarins will only influence the time of onset and the duration of overanticoagulation [17], but not necessarily affect the baseline risk. Second, drugs that interact by inhibiting the isozyme CYP2C9 will affect both acenocoumarol and warfarin [36].

Relative risks varied widely between the different antifungal agents and between the different routes of administration. Displacement of anticoagulant drugs from protein binding sites [18, 37] and inhibition of hepatic metabolism [26-31] has been suggested as potential causes. Plasma protein binding displacement usually plays a minor role compared to other mechanisms [16]. It may be clinically relevant in the elderly, however, in whom plasma protein binding decreases [38]. Antifungal agents, which act through inhibition of the fungal CYP, have the potential to inhibit

human CYP-dependent drug metabolism as well [26, 37, 39]. Ketoconazole and itraconazole, for instance, are potent inhibitors of CYP3A4, whereas fluconazole and miconazole are potent inhibitors of CYP2C9 [30]. CYP2C9 is the principal enzyme that catalyses biotransformation of acenocoumarol and warfarin [40, 41]. Phenprocoumon is also metabolised by CYP2C9 but probably to a lesser extent [36].

Although oral application of miconazole is primarily intended for local oral and gastrointestinal treatment, 25% to 30% is systemically absorbed [42]. As expected, we found that the highest relative risk for miconazole was via the oral route, whereas the relative risk was barely increased after cutaneous application of miconazole. Although the relative risk was increased after vaginal administration of miconazole, the risk increase was not statistically significant. Despite this, it is possible that sometimes sufficient amounts of miconazole are absorbed through this route to interact with coumarins. This would be in line with a case report that suggested that the daily administration of miconazole to an inflamed postmenopausal atrophic vaginal epithelium might lead to potentiation of coumarin anticoagulants [19]. The fact that we found a twice-stronger risk increase by miconazole in patients on acenocoumarol than in patients on phenprocoumon is explained by the fact that phenprocoumon is metabolized to a lesser extent by CYP2C9 than acenocoumarol. Potent inhibitors of CYP2C9, like miconazole, may completely suppress the body clearance of *(S)*-acenocoumarol, converting it from a clinically inactive drug into a potent anticoagulant [40]. Ketoconazol, administered by the cutaneous route, is assumed not to interact with coumarins. Although two case patients were exposed to topical ketoconazole on the index date, the relative risk of overanticoagulation was not increased. For cutaneous clotrimazole and oral itraconazole and nystatin we found a statistically significant increase in relative risks. It should be emphasized, however, that these figures should be cautiously interpreted because they are based on only one case per agent. Our results suggest that terbinafine can be safely coadministered with coumarins. This is in accordance with the literature, according to which terbinafine only inhibits the isozyme CYP2D6 [30]. In addition, it should be emphasized, however, that numbers were low. Hence, the absence of an association does not exclude the possibility of such an interaction in some vulnerable individuals.

Some potential limitations of our cohort study should be considered. Selection bias was probably negligible because we identified all users of coumarin anticoagulants in a defined population and because regular INR monitoring makes it unlikely that cases were missed. Further, information bias is not likely because all data on exposure and outcome were recorded similarly without prior knowledge of our study hypothesis. Potential confounding by gender, age, hepatic dysfunction, hypoalbuminemia, malignancies, hyperthyroidism, hypertension, heart failure, differences in duration of follow-up and INR measurement as scheduled was dealt with in the multivariate analyses. Whether the INR assessment on the index date was earlier than according to plan was taken into consideration because patients who are prescribed a known potentially hazardous combination are more likely to be monitored at short intervals. However, neither adjustment for this nor adjustment for the average number of assessments during

follow-up changed our results. Fever may be a confounding factor, because a hypermetabolic state produced by fever might potentiate the response to coumarin anticoagulants, probably by increasing the catabolism of vitamin K-dependent coagulation factors [2]. There was, however, no statistically significant difference between the frequency of fever in cases on antifungal agents and individuals in the remainder of the cohort who were treated with antifungal agents on the index date. Furthermore, we excluded confounding by indication, because there was no statistically significant difference in the indication for antifungal agents between cases and users of these agents in the remainder of the cohort. A limitation of our study, however, is that numbers were low.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic on coumarins, oral miconazole was especially strongly associated with overanticoagulation. Awareness of these drug interactions and more frequent monitoring during the initial stages of some antifungal drugs in patients on coumarins may minimise the risk of bleeding complications. The concurrent use of miconazole and coumarins should be discouraged.

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Chapter 3.3

Overanticoagulation associated with combined use of lactulose and coumarin anticoagulants

ABSTRACT

Some medical textbooks on drug interactions take note of the potential interaction between laxatives and coumarin anticoagulants, but epidemiological evidence that this interaction is of practical importance is lacking. We conducted a follow-up study in a large population-based cohort to investigate which laxatives are associated with overanticoagulation during therapy with acenocoumarol or phenprocoumon. Of the 1124 patients in the cohort, 351 developed an international normalised ratio $\geq$ 6.0. The only laxative with a moderate but significantly increased relative risk of overanticoagulation was lactulose (relative risk 3.4, 95% confidence interval: 2.2-5.3). In view of the widespread use of lactulose, especially among the elderly, awareness of this potential drug interaction is required.

INTRODUCTION

Coumarin anticoagulants are extensively used for the treatment and long-term prevention of thrombo-embolic diseases [1]. The risk of bleeding, the main complication of coumarin anticoagulants, is influenced by the intensity of anticoagulant therapy [2,3], by the underlying clinical disorders of the patient [3], and by the concomitant use of other drugs [4]. This risk sharply increases when the international normalised ratio (INR) is ≥ 6.0 [5]. Several drugs can affect the INR during coumarin anticoagulant therapy by different mechanisms [4]. Laxatives, which shorten the transit time in the gut, might be expected to decrease the absorption of both vitamin K and coumarin anticoagulants. Despite warnings in the medical literature, there seems to be no epidemiological evidence that this interaction is of any practical importance [6].

Therefore, we conducted a follow-up study in a large population-based cohort to investigate whether laxatives are associated with overanticoagulation during therapy with coumarins.

METHODS

The study cohort consisted of all 1124 patients in one anticoagulation clinic, who were treated with acenocoumarol or phenprocoumon in the study period between April 1, 1991 and December 31, 1998 and for whom there were INR data during their treatment. All cohort members were followed until the first occurrence of an INR ≥ 6.0, the last INR assessment because of the end of their treatment, death or end of the study period, whichever came first. For laxatives for which an association was found, a dose and duration effect was studied. The duration of exposure to a laxative on the day of overanticoagulation was divided into tertiles: ≤ 27 days; > 27 days to ≤ 97 days; and > 97 days. Doses were divided into tertiles: ≤ 7.5 gram; > 7.5 gram to ≤ 12 gram; and > 12 gram. The following baseline patient characteristics were considered as potential determinants for affecting the response of the INR to coumarin anticoagulants: gender, age, hepatic dysfunction, hypoalbuminemia, malignancies, hyperthyroidism, hypertension, heart failure, and low dietary daily intake of vitamin K. Incidence rates were calculated by dividing the number of cases of an INR ≥ 6.0 by the number of days on combined use of a coumarin anticoagulant and a laxative. To assess laxatives which were independently associated with an INR ≥ 6.0, all occurring combinations of a coumarin anticoagulant and a laxative were included separately in a Cox proportional hazards regression model for time-dependent variables to compute relative risks and their 95% confidence intervals (95%CI) [7]. To adjust for potential confounding, cofactors that were associated with an INR ≥ 6.0 in the univariate analysis were included in the multivariate model, in addition to gender and age, if this caused a change in the point estimate of more than 5 percent.

Table 1. Association between overanticoagulation (INR ≥ 6.0) and use of laxatives

Laxative	Patients with INR ≥ 6.0	Total population*	RR_{crude} † (95%CI)	$RR_{adj.}$ ‡ (95%CI)
	n = 351	n = 1124		
Liquid paraffin	1	135	1.2 (0.2-8.9)	0.7 (0.1-5.5)
Colocynthine preparation	4	430	2.3 (0.8-6.1)	1.9 (0.6-6.1)
Psyllium seeds	4	983	1.0 (0.4-2.6)	1.3 (0.3-5.1)
Wheat fibre	6	975	1.5 (0.7-3.4)	2.0 (0.6-6.3)
Lactulose	36	3521	2.6 (1.8-3.6)	3.4 (2.2-5.3)

* In this time-dependent analysis, exposure in case patients and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). Because control patients can be used multiple times, the number of assessments in the reference group is much larger than the number of individuals. Hence, crude relative risks cannot be calculated with the numbers in Table 1.
† If none of the patients with an INR ≥ 6.0 was exposed, P-values are given instead of relative risks.
‡ Adjusted for gender, age, heart failure, low dietary intake of vitamin K.

RESULTS

Of the 1124 patients in the cohort, 351 developed an INR ≥ 6.0 after April 1, 1991. The incidence rate was 6.9 per 10,000 treatment days. Women and older patients had a higher risk of an INR ≥ 6.0. The risk of overanticoagulation was lowest with phenprocoumon. Hepatic dysfunction, malignancies, heart failure and a low dietary daily intake of vitamin K were associated with an increased risk of an INR ≥ 6.0 in the univariate analysis.

Eight different laxatives were used during the study period, of which three were not used in cases. The remaining five laxatives and the relative risks of overanticoagulation are given in Table 1. Fifty-one cases (15%) used laxatives on the index date. Lactulose was the one agent which was univariately as well as multivariately associated with overanticoagulation. After adjustment for confounding factors the relative risk was 3.4 (95%CI: 2.2-5.3).

Stratification by the duration of laxative exposure on the day of overanticoagulation revealed a significantly protective effect of lactulose during the first 27 days of use, the relative risk being 0.5 (95%CI: 0.3-0.8). The relative risk was 1.7 (95%CI: 0.9-3.0) for > 27 days to ≤ 97 days and 2.1 (95%CI: 1.2-3.7) for more than 97 days of lactulose use. A clear dose-effect relationship could not be detected. The relative risks for the subsequent dose levels were 1.7 (95%CI: 0.9-3.1), 2.0 (95%CI: 1.0-4.9) and 1.9 (95%CI: 0.9-4.0).

DISCUSSION

The main finding in this population-based cohort study is that among laxatives, only lactulose was associated with an increased risk of overanticoagulation during coumarin anticoagulant therapy with acenocoumarol or phenprocoumon. Laxatives shorten the transit time in the gut and might be expected to decrease the absorption of both the coumarin anticoagulants and vitamin K. If the absorption of vitamin K is stronger impaired than that of coumarins, overanticoagulation might occur. It seems likely that such effects on the INR would more or

less apply to all laxatives. In addition to that, the long-term oral administration of paraffin may interfere with the absorption of the fat-soluble vitamin K and result in a deficiency of this substance and an increase in INR. The fact that we did not find an association for paraffin may have had two reasons. The first is that there is no real association, all the more as the medical literature mentions only a theoretical possibility that paraffin affects the response to coumarin anticoagulants. The second may be that in our study population paraffin was only used on a short-term basis. With the administration of lactulose the colonic pH will decrease below pH 7.4 [8,9]. This could theoretically have resulted in an increase in the absorption of phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) in the colon [10,11]. This is in line with the observed protective effect during the first month of lactulose therapy. During long-term use, however, another mechanism seems to play a role. We can only speculate about this mechanism, but the biologically most plausible one is that lactulose has its influence on the faecal flora responsible for menaquinone production. Reported effects of lactulose on the faecal flora are conflicting, but counts of the menaquinone-producing bacteria were reported to decrease after lactulose administration [12]. The fact that only lactulose and none of the other laxatives was associated with a significantly increased risk of overanticoagulation could have been due to the lower exposure to these agents in our study. Although our study pertained to the coumarins acenocoumarol and phenprocoumon, it is likely that the results can be extrapolated to warfarin, because here vitamin K plays a similar role.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic on coumarins, lactulose was associated with overanticoagulation. In view of the widespread use of lactulose, especially among the elderly, awareness of this potential drug interaction is required.

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Chapter 3.4

Allelic variants of cytochrome P450 2C9 modify the interaction between NSAIDs and coumarin anticoagulants

ABSTRACT

Introduction: Cytochrome P450 2C9 (CYP2C9) plays a key role in the metabolism of coumarin anticoagulants and nonsteroidal anti-inflammatory drugs (NSAIDs). Because CYP2C9 is a genetically polymorphic enzyme, genetic variability could play an important role in the potential interaction between NSAIDs and coumarins. We investigated whether NSAIDs were associated with overanticoagulation during therapy with coumarins, and evaluated the effect of the CYP2C9 polymorphisms on this potential interaction.

Methods: We conducted a population-based cohort study among patients of an anticoagulation clinic who were treated with acenocoumarol or phenprocoumon between April 1, 1991 and May 31, 2003 and whose CYP2C9 status was known. Patients were followed until an international normalised ratio (INR) $\geq$ 6.0, end of treatment, death or end of the study. Proportional hazards regression analysis was used to estimate the risk of an INR $\geq$ 6.0 in relation to concomitant use of a coumarin anticoagulant and NSAIDs after adjustment for several potentially confounding factors. In order to study effect modification by CYP2C9 genotype, stratified analyses were performed for wild type patients and patients with a variant genotype.

Results: Of the 973 patients in the cohort, 415 had an INR $\geq$ 6.0. Several NSAIDs increased the risk of overanticoagulation. The risk of overanticoagulation was almost fourfold in coumarin-treated patients on NSAIDs with a variant allele.

Conclusions: Several NSAIDs increased the risk of overanticoagulation. Allelic variants of CYP2C9 modified this risk. Awareness of this drug interaction and more frequent monitoring of the INR of patients receiving these drugs are warranted.

INTRODUCTION

Coumarin anticoagulants are associated with life-threatening drug-drug interactions because they exhibit a narrow therapeutic range, high protein binding and cytochrome P450 (CYP)-dependent, capacity-limited hepatic clearance [1]. A considerable number of interactions with coumarin anticoagulants have been reported and summarised in a number of comprehensive reviews [1-5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) increase the risk of bleeding in patients on coumarin anticoagulants, mainly due to inhibition of platelet aggregation [1, 2]. This pharmacodynamic interaction, however, is not accompanied by a change in prothrombin time as measured by the international normalised ratio (INR) [6, 7]. The cytochrome P450 2C9 (CYP2C9) enzyme plays a key role in the metabolism of coumarin anticoagulants and NSAIDs [8]. This raises the possibility of a pharmacokinetic drug interaction resulting in an increased INR. CYP2C9 is a genetically polymorphic enzyme. Allelic variants of CYP2C9, CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu), code for enzymes with approximately 12% and 5% of the enzymatic activity of the wild type genotype CYP2C9*1 (Arg144/Ile359) respectively [9-11]. Therefore, genetic variability might play an important role in this potential drug interaction.

In view of the risks of an increased INR, such as bleeding, we investigated whether NSAIDs were associated with overanticoagulation during therapy with coumarin anticoagulants and evaluated the effect of the CYP2C9 polymorphisms on this potential interaction.

METHODS

Setting

Data were obtained from the regional outpatient anticoagulation clinic, and from the Rotterdam Study, a prospective population-based cohort study in which 7983 inhabitants aged 55 years or over participate since 1990. The Medical Ethics Committee of the Erasmus MC approved the study, and written informed consent was obtained from all participants. The rationale and design of this study have been described elsewhere [12]. All participants were extensively interviewed and investigated at baseline. Blood was taken for the assessment of several laboratory values. The anticoagulation clinic in the Rotterdam-Rijnmond area monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. Prothrombin times are monitored every one to six weeks by reference to the INR, depending on the stability of the anticoagulant level. Doses are adjusted on the basis of the target range of the INR of the patient by computerised dose calculations. We used data from April 1, 1991 through May 31, 2003 that were linked to the Rotterdam Study. More than 99% of participants fill their drug prescriptions at seven regional pharmacies, which are fully computerised. Complete data on drug use were available as of 1 January 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code [13], the filling date, the total

amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs.

Cohort and outcome definition

The study cohort consisted of all 973 patients in the Rotterdam Study who were treated with acenocoumarol or phenprocoumon between April 1, 1991 and May 31, 2003, for whom INR data from their treatment history were available, and whose CYP2C9 status was known. The start date of April 1 was chosen to ensure that at least 3 months of medication history from the pharmacy was available for every cohort member. All cohort members were followed as of April 1, 1991 for prevalent users of coumarins and from their first INR assessment for incident users. Both groups were followed until the first occurrence of an INR $\geq$ 6.0 (at which level the risk of bleeding sharply increases), the last INR assessment because of the end of their treatment, death or end of the study period, whichever came first. The date on which an INR $\geq$ 6.0 was encountered was defined as the index date.

Genotyping

Genotyping for the CYP2C9*2 and CYP2C9*3 allele variants was performed by using polymerase chain reaction followed by restriction enzyme digestion analysis [14]. All CYP2C9*2 and CYP2C9*3 heterozygote and homozygote variants were reanalyzed. Patients in whom neither CYP2C9*2 nor CYP2C9*3 alleles were detected were regarded as wild type.

Cofactors

The following baseline patient characteristics were considered as potential confounders or effect modifiers: gender, age, target INR level, hepatic dysfunction (defined as serum aminotransferases > 2x the upper level of normal), hypoalbuminemia ($\leq$ 35 g/l), malignancies, hyperthyroidism, hypertension (systolic blood pressure $\geq$ 140 mm Hg and/or diastolic blood pressure $\geq$ 90 mm Hg or use of antihypertensives), heart failure, low dietary intake of vitamin K (< 1 µg/kg/day) and CYP2C9 genotype. In addition, we considered use of antibacterial drugs on the index date, duration of follow-up and whether the INR measurement on the index date was earlier than according to the INR monitoring scheme. Furthermore, we gathered data on the indication for NSAIDs from medical records. We did this validation for all cases and a random sample from the remainder of the cohort who all had been treated with NSAIDs on the index date.

Statistical analysis

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by using a χ^2-test. Incidence rates of overanticoagulation were calculated by dividing the number of cases of an INR $\geq$ 6.0 by the number of days on combined use of a coumarin anticoagulant and an NSAID. To assess NSAIDs which were independently associated with an INR $\geq$ 6.0, all

occurring combinations of a coumarin anticoagulant and an NSAID were included separately in a Cox proportional hazards regression model for time-dependent variables to compute relative risks and their 95% confidence intervals (95%CI) [15]. The model compares exposure to this combination on the index date of each case with that of all other non-censored subjects in the cohort on the same date as the case. To adjust for potential confounding, cofactors that were associated with an INR ≥ 6.0 in the univariate analysis were included in the multivariate model if this caused a change in the point estimate of more than 5 percent. In order to study effect modification, patients were stratified as wild type (CYP2C9*1/*1 homozygotes) and variant (1 or 2 of the mutant alleles CYP2C9*2 or CYP2C9*3). These analyses were also performed for the separate coumarins.

RESULTS

Of the 973 patients in the cohort, 415 had an INR ≥ 6.0 at an incidence rate of 5.1 per 10,000 treatment days. Baseline characteristics of patients with an INR ≥ 6.0 and the total cohort are shown in Table 1. Women and older patients had a significantly higher risk of an INR ≥ 6.0. There were 668 patients (68.7%) with the wild type genotype, and 305 (31.3%) with a variant genotype. The frequencies of the CYP2C9*2 and CYP2C9*3 alleles were 13.5% and 4.1%, respectively. Allele and genotype proportions were in Hardy-Weinberg equilibrium. All patients were of Caucasian origin. None of the individual genotypes had a statistically significantly higher risk of overanticoagulation than the wild type genotype. The risk of overanticoagulation was lowest with phenprocoumon. Patients targeted at higher INR levels had a higher risk of overanticoagulation, but the differences with the lowest target level were not statistically significant. Hepatic dysfunction, hypoalbuminemia, malignancies and heart failure were associated with an increased risk of an INR ≥ 6.0 in the univariate analysis. Patients who used antibacterial drugs on the index date had a significantly higher risk of overanticoagulation (RR 4.85, 95%CI: 3.57-6.58). There was no difference in duration of follow-up between patients with an INR ≥ 6.0 and the total cohort (mean ± SD, 763 ± 737 days). The INR measurement on the index date was earlier than scheduled in 16.7% of the cases and in 10.9% of the total cohort ($p = 0.001$).

Fourteen different NSAIDs were used during the study period, of which 6 were not used in patients with an INR ≥ 6.0 (Table 2). Thirty-five cases (8.4%) used NSAIDs on the index date (Table 1). Incidence rates for the individual NSAIDs are presented in Table 2. Four NSAIDs were univariately as well as multivariately associated with an INR ≥ 6.0. There was no difference in the indication for NSAIDs between cases and the remainder of the cohort (mainly osteoarthritis).

Because numbers per individual NSAID were too low to study effect modification by CYP2C9 genotype, exposure to NSAIDs was pooled. Table 3 shows the effect of the CYP2C9 genotype on the drug interaction between coumarin anticoagulants and NSAIDs. The interaction term

Table 1. Baseline characteristics of patients with an INR ≥ 6.0 and the total cohort

	Patients with INR ≥ 6.0	Total cohort	RR*	(95%CI)
	n = 415	n = 973		
Gender				
Male	206 (49.6%)	475 (48.8%)	1.00	Reference
Female	209 (50.4%)	498 (51.2%)	1.70	(1.40-2.06)
Age (yrs, mean (SD))	72 (7)	71 (8)	1.03	(1.02-1.05)
55-64	75 (18.1%)	222 (22.8%)	1.00	Reference
65-74	191 (46.0%)	438 (45.0%)	1.19	(0.91-1.55)
75-84	132 (31.8%)	272 (28.0%)	1.64	(1.23-2.19)
≥ 85	17 (4.1%)	41 (4.2%)	2.47	(1.45-4.22)
Genotype				
*1/*1	282 (67.9%)	668 (68.7%)	1.00	Reference
*1/*2	88 (21.2%)	205 (21.1%)	1.08	(0.85-1.37)
*2/*2	11 (2.7%)	20 (2.1%)	0.98	(0.53-1.78)
*1/*3	27 (6.5%)	63 (6.5%)	1.46	(0.99-2.18)
*2/*3	7 (1.7%)	17 (1.7%)	1.62	(0.77-3.44)
*3/*3	-	-	-	
Type of anticoagulant				
Acenocoumarol	349 (84.1%)	825 (84.8%)	1.00	Reference
Phenprocoumon	66 (15.9%)	148 (15.2%)	0.60	(0.46-0.78)
Target level				
2.0-2.5 INR	1 (0.2%)	25 (2.6%)	1.00	Reference
2.5-3.5 INR	104 (25.1%)	344 (35.3%)	4.64	(0.65-33.4)
3.0-4.0 INR	310 (74.7%)	603 (62.0%)	4.98	(0.69-35.7)
3.5-4.5 INR	-	1 (0.1%)	-	
Hepatic dysfunction	6 (1.4%)	12 (1.2%)	4.41	(1.93-10.1)
Hypoalbuminemia	1 (0.2%)	2 (0.2%)	21.8	(2.73-175)
Malignancies	58 (14.0%)	114 (11.7%)	1.97	(1.49-2.61)
Hyperthyroidism	14 (3.4%)	29 (3.0%)	1.63	(0.95-2.79)
Hypertension	183 (44.1%)	385 (39.6%)	1.01	(0.82-1.21)
Heart failure	130 (31.3%)	209 (21.5%)	1.84	(1.50-2.27)
Low intake vitamin K	9 (2.2%)	17 (1.7%)	1.85	(0.95-3.60)

* Univariate analyses of relative risks were performed with a Cox proportional-hazards model. Relative risks cannot be calculated with the numbers in this table because controls may later become cases.

between CYP2C9 genotype and the pooled exposure was highly statistically significant (p = 0.009). The adjusted relative risk of the pooled exposure was 1.62 (95%CI: 1.03-2.57) in patients with the wild type genotype, and 3.76 (95%CI: 2.09-6.79) in patients with a variant genotype (Table 3). Stratified analyses by the type of anticoagulant revealed a statistically significant interaction term between exposure and CYP2C9 genotype for patients on acenocoumarol (p = 0.02) but not for phenprocoumon (p = 0.14).

Table 2. Association between overanticoagulation (INR ≥ 6.0) and use of NSAIDs

NSAID	Cases	Cohort*	IR†	RR_{crude} (95%CI)‡	$RR_{adj.}$§ (95%CI)
	n=415	n=973			
Celecoxib	0	16	-	P=0.78	
Diclofenac	15	1143	2.6	2.36 (1.40-3.97)	2.25 (1.33-3.80)
Diclofenac/misoprostol	1	174	1.9	0.73 (0.10-5.27)	0.31 (0.04-2.27)
Flurbiprofen	0	100	-	P=0.66	
Ibuprofen	6	402	1.7	2.64 (1.17-5.96)	2.47 (1.09-5.61)
Indometacin	1	52	0.6	2.73 (0.37-20.1)	1.61 (0.22-11.9)
Ketoprofen	0	26	-	P=0.68	
Meloxicam	0	11	-	P=0.85	
Nabumetone	0	96	-	P=0.64	
Naproxen	8	591	2.2	2.49 (1.23-5.03)	2.44 (1.20-4.97)
Piroxicam	1	59	5.8	1.87 (0.25-13.8)	1.98 (0.25-15.5)
Rofecoxib	1	24	20.2	3.99 (0.53-30.2)	4.67 (0.61-35.7)
Sulindac	2	26	60.8	14.2 (3.34-60.2)	15.4 (3.47-68.6)
Tiaprofenic acid	0	10	-	P=0.88	

* In this time-dependent analysis, exposure in case patients and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). Because control patients can be used multiple times, the number of assessments in the reference group is much larger than the number of individuals. Hence, crude relative risks cannot be calculated with the numbers in Table 2.
† Expressed as the number of patients with an INR ≥ 6.0 per 10,000 days on a regimen of combined use of a coumarin and an NSAID.
‡ If none of the cases was exposed, P-values were given instead of relative risks.
§ Adjusted for gender, age, hepatic dysfunction, malignancies, heart failure, CYP2C9 genotype, and use of antibacterial drugs.

Table 3. Association between overanticoagulation (INR ≥ 6.0) and NSAIDs stratified by CYP2C9 genotype

	$RR_{adj.}$* (95%CI)
Total study population	
CYP2C9 wild type; NSAIDs (–)	1.00 (reference)
CYP2C9 variant allele; NSAIDs (–)	1.12 (0.90-1.39)
CYP2C9 wild type; NSAIDs (+)	1.62 (1.03-2.57)
CYP2C9 variant allele; NSAIDs (+)	2.14 (1.50-3.06)
*P-value for statistical interaction CYP2C9*NSAIDs*	*P=0.009*
Population with CYP2C9 wild type	
CYP2C9 wild type; NSAIDs (–)	1.00 (reference)
CYP2C9 wild type; NSAIDs (+)	1.62 (1.03-2.57)
Population with CYP2C9 variant allele	
CYP2C9 variant allele; NSAIDs (–)	1.00 (reference)
CYP2C9 variant allele; NSAIDs (+)	3.76 (2.09-6.79)

* Adjusted for gender, age, hepatic dysfunction, malignancies, heart failure, and use of antibacterial drugs.

DISCUSSION

We found that several NSAIDs increased the risk of overanticoagulation, and that allelic variants of CYP2C9 modified this risk. The fact that we found an association with acenocoumarol but not with phenprocoumon may be explained by the fact that the CYP2C9 enzyme plays a minor role

in the metabolism of phenprocoumon [16]. It should be emphasized, however, that the number of patients on phenprocoumon in this study was relatively small.

Unfortunately, there were too little exposed cases to study effect modification by CYP2C9 genotype for the individual NSAIDs and for the separate genotypes. Because in vitro investigations and clinical studies have identified many NSAIDs as substrates of CYP2C9 [17-27], we pooled all NSAIDs in one exposure category. When we restricted our analyses to NSAIDs that are known CYP2C9 substrates we found, however, comparable risk estimates.

We are aware of only one similar but smaller study that suggested that a variant CYP2C9 genotype did not influence the NSAID-acenocoumarol interaction [28]. The sample size of that study was, however, too small to study the effect of the CYP2C9 polymorphisms on this drug interaction properly and no adjustments were made for potentially confounding factors.

Some potential limitations of our cohort study should be considered. Selection bias was probably negligible because we identified all users of coumarin anticoagulants in a defined population. Furthermore, our study population was in Hardy-Weinberg equilibrium, suggesting that no selection has occurred among genotypes, which might otherwise have explained the observed association. Information bias may have occurred by over-the-counter (OTC) use of NSAIDs. This would lead, however, to a conservative estimation of the association. Moreover, it would not influence the interaction between CYP2C9 polymorphisms and NSAIDs. We adjusted for all known potential confounders and excluded confounding by indication, because there was no statistically significant difference in the indication for NSAIDs between cases and users of these agents in the remainder of the cohort. Whether the INR assessment on the index date was earlier than according to plan was taken into consideration because patients who are prescribed a known potentially hazardous combination are more likely to be monitored at short intervals. Adjustment for this did, however, not change our results. We did not adjust our results for NSAID dosages nor for the concomitant use of other drugs than antibacterial drugs, because there were no reasons to assume that other drugs would act as a confounder.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic using acenocoumarol or phenprocoumon, several NSAIDs were associated with overanticoagulation. The risk of overanticoagulation was modified by allelic variants of CYP2C9. Awareness of this drug interaction and more frequent monitoring of the INR of patients receiving these drugs are warranted. As warfarin is also mainly metabolized by CYP2C9, it is likely that our results also apply to warfarin.

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Chapter 4

Disease states affecting the coumarin anticoagulant level

Chapter 4.1

The risk of overanticoagulation in patients with heart failure on coumarin anticoagulants

ABSTRACT

Objective: Heart failure has been identified as a risk factor for increased coumarin anticoagulant responsiveness in several small-scale experiments. Epidemiological studies quantifying the risk of overanticoagulation by heart failure in a non-selected population on coumarins are scarce. Therefore, we investigated whether patients with heart failure have an increased risk of overanticoagulation and determined the effect of incident heart failure on coumarin dose requirements.

Methods: We performed a cohort study in all patients from an outpatient anticoagulation clinic treated with acenocoumarol or phenprocoumon between January 1, 1990 and January 1, 2000. All cohort members were followed until the first occurrence of an international normalised ratio (INR) $\geq$ 6.0, the last INR-assessment, death, loss to follow up, or end of the study period.

Results: Of the 1077 patients in the cohort, 396 developed an INR $\geq$ 6.0. The risk of overanticoagulation was 1.66 (95%CI: 1.33-2.07) for cases of prevalent heart failure and 1.91 (95%CI: 1.31-2.79) for incident cases. The decrease in dose requirements in patients with incident heart failure showed a significant trend from the 5th INR measurement preceding the incident heart failure date to the 3rd measurement after this date.

Conclusion: Heart failure is an independent risk factor for overanticoagulation. Therefore, patients with heart failure should be closely monitored to prevent potential bleeding complications.

INTRODUCTION

Coumarin anticoagulants are used in the primary and secondary prophylaxis of thromboembolic disease [1]. They inhibit the production of the vitamin K-dependent coagulation factors by the liver [2]. Inherent to their mode of action and narrow therapeutic range, hemorrhage is the most common adverse reaction to coumarin anticoagulants. The risk of hemorrhage is strongly associated with the intensity of anticoagulation and sharply increases when the international normalised ratio (INR) $\geq$ 6.0 [3, 4]. A number of comorbid conditions are suspected to enhance the response to coumarin anticoagulants [5, 6]. In some small-scale experiments in groups up to 30 patients in the late 40's of the last century, heart failure has been identified as a risk factor for increased coumarin responsiveness [7, 8]. The mechanism has not been fully elucidated but it is speculated that increases in coumarin responsiveness are associated with hepatic congestion and redistribution of body water [6-10]. Epidemiological studies quantifying the risk of overanticoagulation by heart failure in a non-selected population on coumarins are scarce. A recent case-control study suggested that patients with heart failure had an increased risk of an INR $\geq$ 6.0 [11]. In that study, however, the presence of chronic comorbidities was only based on diagnoses of general practitioners and no cases of incident heart failure were included. Therefore, we conducted a follow-up study in a large population-based cohort among outpatients of an anticoagulation clinic on acenocoumarol or phenprocoumon. We studied the association between heart failure and overanticoagulation and determined the effect of heart failure on the coumarin dosage.

METHODS

Setting

Data were obtained from the Rotterdam Study and from the regional outpatient anticoagulation clinic. The Rotterdam Study is a prospective population-based cohort study in 7983 subjects of 55 years and older and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre [12]. The baseline examination was conducted between 1990 and 1993. Participants were visited at home for a standardized questionnaire and were subsequently examined at the research centre. At baseline, information was obtained on several characteristics, including age, gender, smoking, body mass index (BMI), medication use, blood pressure, and verified history of heart failure. In the Rotterdam Study, participants are continuously monitored for major events that occur during follow-up, including heart failure, through automated linkage with files from general practitioners. All available information on these events is copied from the medical records for verification of the diagnosis. Furthermore, all drug prescriptions dispensed to participants by automated pharmacies are routinely stored in the database since January 1, 1991. Information on vital status is obtained regularly from municipal health authorities in

Rotterdam and from the general practitioners in the study district, and was complete for all participants until January 1, 2000.

The anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant is made by the physician. The optimal target range of coumarin anticoagulant therapy, as recommended by the Federation of Dutch Thrombosis Centers, lies between 2.5 and 3.5 INR or between 3.0 and 4.0 INR, depending on the indication for treatment. Some patients are targeted at a level between 2.0 and 2.5 INR because of contraindications. Prothrombin times are monitored each one to six weeks by reference to the INR, dependent on the stability of the anticoagulant level. Doses are adjusted on the basis of the target range of the INR of the patient by computerised dose calculations. All data on dosing, laboratory-, and clinical data as of 1984 are fully computerised. For this study, data were used from January 1, 1990 through January 1, 2000.

Cohort and outcome definition

The study cohort consisted of all participants of the Rotterdam Study who were treated with acenocoumarol or phenprocoumon in the study period between the baseline visit of the Rotterdam Study and January 1, 2000 and for whom INR data from their treatment history were available. If a patient had multiple treatment episodes during the study period, only the first episode after the baseline examination was considered. The cohort included patients on coumarin anticoagulants without heart failure, patients on coumarins with prevalent heart failure at baseline and patients on coumarins who developed heart failure during the study period (incident cases). All cohort members were followed up as of their baseline examination for patients without heart failure and for cases of prevalent heart failure, and from the date of incident heart failure for incident cases until the earliest of an INR $\geq$ 6.0, death, loss to follow up, or January 1, 2000. In case the date of incident heart failure was not during a treatment episode with coumarins, follow-up started at the first day of the next treatment episode and heart failure was classified as prevalent. The index date was defined at that point in time on which one of the endpoints occurred for a participant of this study. The effect of heart failure on the coumarin dosage was determined by calculating the average week dosage per INR measurement for the subsequent measurements after start of the follow up. This was separately done for prevalent and incident cases of heart failure and for the rest of the cohort, and for both acenocoumarol and phenprocoumon. For the incident cases of heart failure, for which the follow-up started at the date of incident heart failure, we also studied the course of the coumarin dosage from the 10th INR measurement preceding this date.

Heart failure assessment

Assessment of prevalent heart failure at baseline has been described in detail earlier [13]. Briefly, a validated score was used, similar to the definition of the European Society of Cardiology [14]. This score was based on the presence of at least two symptoms suggestive of heart failure or

treatment for heart failure, in combination with objective evidence of cardiovascular disease. This score was, however, not implemented from the first beginning of the Rotterdam Study, but was subsequently added. Consequently, this information was obtained in only 5440 participants. In addition, prevalent heart failure cases were obtained through a database containing hospital discharge diagnoses from all hospitals in the Rotterdam area. Furthermore, all medical records were screened in retrospect for the occurrence of heart failure in the majority of participants. With these three methods, information on prevalent heart failure was available for all participants.

Cases of incident heart failure were obtained by continuously monitoring participants of the Rotterdam Study for the occurrence of heart failure during follow-up through automated linkage with files from general practitioners. All available data on these events, such as hospital discharge letters and notes from general practitioners, were copied from the medical records. Apart from this systematic follow-up procedure, we used verified hospital discharge diagnoses for case finding, gathered from all hospitals in the Rotterdam area as described above. The diagnosis of heart failure was classified as definite, probable, possible or unlikely. Definite heart failure was defined as a combination of heart failure, such as breathlessness at rest or during exertion, ankle oedema and pulmonary reputations, confirmed by objective evidence of cardiac dysfunction (chest X-ray, echocardiography). This definition is in accordance with the criteria of the European Society of Cardiology [14]. Probable heart failure was defined as heart failure diagnosed by a general practitioner, with at least two typical symptoms suggestive of heart failure, and at least 1 of the following: history of cardiovascular disease (e.g. myocardial infarction, hypertension), response to treatment for heart failure, or objective evidence of cardiac dysfunction, while symptoms could not be attributed to another underlying disease, such as chronic obstructive pulmonary disease. Two research physicians independently classified all information on potential heart failure events. If there was disagreement, a consensus was reached in a separate session. Finally, a cardiologist verified all probable and possible cases, and all cases in which the two physicians could not reach consensus. If the cardiologist disagreed with the research physicians, the cardiologist's judgement was considered decisive. Only definite and probable cases were included in the analyses.

Cofactors

The following baseline patient characteristics were considered as potential determinants for affecting the response of the INR to coumarin anticoagulants: gender, age, CYP2C9 genotype, hepatic dysfunction (defined as serum aminotransferases > 2x the upper level of normal), hypoalbuminemia (≤ 35 g/l), malignancies, hyperthyroidism, hypertension (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensives) and low dietary intake of vitamin K (< 1 µg/kg/day). In addition, we considered the type of anticoagulant, the indication for therapy, the target INR level, the number of visits to the anticoagulation clinic during the follow-up period, and the use of thiazides or non-steroidal

anti-inflammatory drugs (NSAIDs) on the index date as potential confounding factors. Separate analyses were performed for prevalent and incident cases of heart failure.

Statistical analysis

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium (HWE) by using a χ^2-test. Independent-sample t-tests and Pearson's chi-square were used to compare baseline characteristics between prevalent or incident heart failure cases and patients without heart failure. Incidence rates of overanticoagulation were calculated by dividing the number of cases of an INR $\geq$ 6.0 by the number of days on a coumarin anticoagulant. The association between heart failure and overanticoagulation was evaluated using Cox proportional hazards regression analysis to estimate relative risks (RR) and 95% confidence intervals (95%CI). To adjust for potential confounding, cofactors were included in the model, in addition to age and gender, if the point estimate changed by more than 5% upon inclusion of the cofactor in the model. For missing data on categorical covariates, we used a missing value indicator, whereas for missing data on continuous covariates, we used the median value of the respective value as calculated from the total sample. To evaluate the effect of incident heart failure on the coumarin dosage, a trend test was performed using a linear regression model with the mean dosage as outcome variable. For all statistical analyses p-values below 0.05 were considered statistically significant. All statistical analyses were performed with SPSS version 11.0.1 (SPSS Inc., Chicago, USA).

RESULTS

A total of 1077 individuals on coumarin anticoagulants were included in our study population (Table 1). The mean age of these patients was almost 72 years, and 47.4% of the patients were men. All patients were of Caucasian origin. There were 636 patients (59.1%) with the wild type CYP2C9 genotype (CYP2C9*1/*1 homozygotes), and 323 (30.0%) with a variant genotype (1 or 2 of the mutant alleles CYP2C9*2 or CYP2C9*3). Allele and genotype proportions were in Hardy-Weinberg equilibrium. There were 915 acenocoumarol-treated patients (85.0%), and 162 patients (15.0%) who used phenprocoumon. Twenty-five patients (2.3%) were targeted at an INR between 2.0 and 2.5, 368 patients (34.2%) at an INR between 2.5 and 3.5, and 684 patients (63.5%) at an INR between 3.0 and 4.0. Patients had a median of 29 INR assessments during a median follow-up time of 245 days (0.7 years).

There were 234 cases of prevalent and 66 cases of incident heart failure identified during the study period. Patients with prevalent heart failure were significantly older and more likely to be male than patients without heart failure. They used more often phenprocoumon, were more often targeted at the highest INR level and had their INR more frequently measured. Patients with incident heart failure were significantly older, were more often targeted at the highest

Table 1. Characteristics of the study population

Variable	Number of patients (n = 1077)
Gender	
Male	511 (47.4%)
Female	566 (52.6%)
Age, average (SD)	71.8 (7.9) years
CYP2C9 genotype*	
Wild type genotype†	636 (59.1%)
Variant genotype‡	323 (30.0%)
Type of anticoagulant	
Acenocoumarol	915 (85.0%)
Phenprocoumon	162 (15.0%)
Indication	
Prophylaxis venous thrombosis	227 (21.1%)
Treatment of venous thrombosis	368 (14.0%)
Treatment or prophylaxis of arterial thrombosis	673 (62.4%)
Prosthetic heart valves	25 (2.3%)
Target INR level	
2.0-2.5 INR	25 (2.3%)
2.5-3.5 INR	368 (34.2%)
3.0-4.0 INR	684 (63.5%)
Time between visits (d ± SD)	11.9 (± 16.3)
Hepatic dysfunction	12 (1.1%)
Hypoalbuminemia	2 (0.2%)
Malignancies	123 (11.4%)
Hyperthyroidism	37 (3.4%)
Hypertension	422 (39.2%)
Low intake of vitamin K	16 (1.5%)
Use of thiazides§	4 (0.4%)

* Totals do not add up to 100% because of missing genotypes.
† CYP2C9*1/*1 homozygotes.
‡ Patients with one or more of the variant alleles CYP2C9*2 or CYP2C9*3.
§ Assessed by reference to the index date.

INR level and were more likely to have hypoalbuminemia than patients without heart failure. Patients without heart failure used coumarins more often for prophylaxis and treatment of venous thrombosis, while patients with prevalent and incident heart failure had more often an arterial indication for coumarin anticoagulant therapy.

During the study period, 396 of the 1077 individuals (37%) had an INR ≥ 6.0. From the 234 prevalent cases of heart failure 131 individuals (56%) experienced an INR ≥ 6.0, and from the 66 incident heart failure cases 32 individuals (48%) had overanticoagulation. Table 2 presents relative risk estimates for the association between prevalent and incident heart failure and overanticoagulation. Prevalent and incident heart failure were both univariately as well as after adjustment for confounding factors associated with an increased risk of overanticoagulation.

Table 2. Relative risks for the association between heart failure and overanticoagulation

	N	Events	IR*	RR_{crude} (95%CI)	$RR_{adj.}$† (95%CI)
Patients without heart failure	777	233	5.19	1.00 (reference)	1.00 (reference)
Prevalent heart failure	234	131	9.82	1.84 (1.48-2.28)	1.66 (1.33-2.07)
Incident heart failure	66	32	11.78	1.97 (1.36-2.86)	1.91 (1.31-2.79)

* The incidence rate is expressed as the number of cases of overanticoagulation per 10,000 days on a coumarin anticoagulant.
† Adjusted for gender, age, and target INR level.

In Figure 1 the mean weekly dosage of acenocoumarol is shown for the subsequent INR measurements after start of the follow-up. Patients with prevalent and incident heart failure used lower dosages than patients without heart failure, in spite of the higher target INR levels for patients with heart failure. Patients with incident heart failure had even lower acenocoumarol dose requirements than patients with prevalent heart failure. Because this difference did not disappear in the course of time, this probably reflects the difference in indication and target INR level. For phenprocoumon we saw more or less the same picture. For patients with incident heart failure the decrease in dosage started on average at the 5th INR measurement preceding the incident date and lasted until the 3rd INR measurement after the incident date. A trend test revealed a significant dosage decrease between the subsequent INR measurements of 0.23 mg per week for acenocoumarol (p for trend <0.001) and 0.34 mg per week for phenprocoumon (p for trend <0.001).

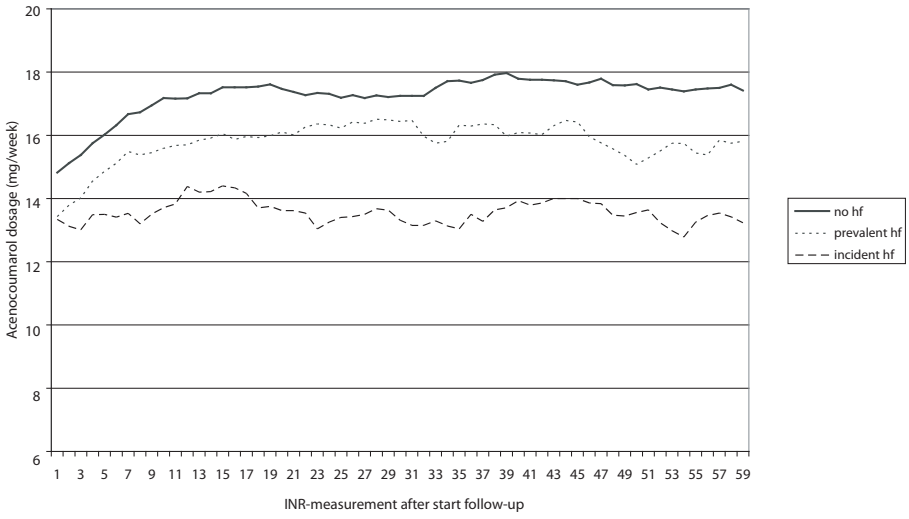

Figure 1. Course of the mean acenocoumarol dosage over time. Hf, heart failure

DISCUSSION

The current study identifies heart failure as an independent risk factor for excessive anticoagulation under everyday circumstances. Patients with heart failure had a 1.5 to twofold increased risk of an INR ≥ 6.0. Our results are in accordance with the study of Penning-van Beest, who found an OR of 1.6 (95%CI: 1.04-2.6) in stable condition and an OR of 3.0 (95%CI: 0.8-12.0) in case of a relapse [11].

This increased coumarin responsiveness is assumed to be due to impairment of liver function resulting from congestion [6-9]. Patients with heart failure were noted to have an increased response as hepatic congestion developed [6, 9], and the responsiveness decreased on relief of the congestion by corrective cardiac surgery [15] or use of diuretics [16]. Our data also indicate that coumarin responsiveness is already slowly increasing during the weeks preceding the incident heart failure date, probably due to increasing hepatic congestion. It is speculated that the determinants of increased coumarin responsiveness might chiefly be pharmacodynamic (associated with impaired clotting factor synthesis) rather than pharmacokinetic (associated with decreased hepatic coumarin clearance) [10, 16]. The hypothrombinaemic effect will be even larger due to the redistribution of body water in heart failure patients and consequent accumulation of unbound coumarin anticoagulant in the vicinity of hepatic receptor sites [10].

The clinical implication of these findings lies in the possibility of prevention or early detection of excessive anticoagulation, and thus of hemorrhagic complications, by paying special attention to this risk factor when monitoring anticoagulant therapy. Patients with heart failure should therefore be closely monitored for signs of excess anticoagulation and fluid overload and it should be noted whether patients are taking any drugs associated with fluid retention (e.g. vasodilators, NSAIDs).

In our study, selection bias was probably negligible as we identified all users of oral anticoagulants in a defined population and because regular INR monitoring makes it unlikely that cases were missed. Also, information bias is not likely as all data on heart failure and coumarin anticoagulant dosages were recorded similarly without prior knowledge of our study hypothesis. Potential confounding by gender, age, CYP2C9 genotype, hepatic dysfunction, hypoalbuminemia, malignancies, hyperthyroidism, hypertension, low dietary intake of vitamin K, type of anticoagulant, indication for therapy, target INR level, time between the INR measurements, and use of thiazides and NSAIDs was dealt with in the multivariate analyses. Use of thiazide diuretics on the index date was taken into consideration because current use of these drugs was found to increase the bleeding risk of oral anticoagulant therapy by 5.2% [17].

In conclusion, heart failure is an independent risk factor for overanticoagulation. Patients with heart failure should therefore be closely monitored for signs of excess anticoagulation and fluid overload to prevent potential bleeding complications.

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Chapter 5

Dietary factors influencing the coumarin anticoagulant level

Chapter 5.1

Deficient dietary intake of vitamin K is associated with an increased risk of overanticoagulation

ABSTRACT

A dietary intake of vitamin K of 1 µg/kg body weight per day is required for normal functioning of coagulation factors. Possibly, a deficient intake of vitamin K is associated with overanticoagulation. We performed a population-based cohort study in a sample of the Rotterdam Study to study whether patients with a deficient dietary intake of vitamin K have an increased risk of overanticoagulation (international normalised ratio (INR) ≥ 6.0). The study cohort consisted of all participants of whom dietary intake data have been collected and who were treated with coumarin anticoagulants in the study period from the baseline visit of the Rotterdam Study (1990-1993) through December 31, 1998. All cohort members were followed from their baseline visit of the Rotterdam Study until the first occurrence of an INR ≥ 6.0, the last INR assessment during the study period, death or end of the study period. The intake of vitamin K was calculated from the total diet using data on concentrations of vitamin K_1 and vitamin K_2 in foods. An intake of vitamin K below 1 µg/kg body weight per day was considered deficient. Of the 772 patients in the cohort, 227 developed an INR ≥ 6.0 during the study period. The number of patients in the total cohort with a deficient dietary intake of vitamin K was 12 (1.6%). Of the cases, seven patients (3.1%) had a deficient dietary intake of vitamin K. The adjusted relative risk of overanticoagulation associated with a deficient dietary intake of vitamin K was 9.6 (95% confidence interval: 4.0-23.0). To minimize the risk of bleeding complications, patients on coumarin anticoagulant therapy should be advised to consume vitamin K-rich foods such as green, leafy vegetables.

INTRODUCTION

Coumarin anticoagulants are widely used in the prevention of venous and arterial thromboembolism [1]. These drugs induce anticoagulation by antagonizing vitamin K, thereby impairing the biological activity of the vitamin K-dependent coagulation factors II, VII, IX and X [2]. Hemorrhage is the most common adverse reaction to coumarin anticoagulants. Its risk is strongly associated with the intensity of anticoagulation and sharply increases when the international normalised ratio (INR) is $\geq$ 6.0 [3, 4]. For normal functioning of coagulation factors, a habitual dietary intake of vitamin K of 1 µg/kg body weight per day is required [5, 6]. Considering the mode of action of coumarin anticoagulants, it is obvious that a low dietary intake of vitamin K will require a low-normal daily dose of coumarins. Because coumarin anticoagulant therapy is regularly monitored and the dose of coumarins is adjusted in an individual on the basis of the INR, however, there is no reason to assume that a patient's low habitual dietary intake of vitamin K is associated with an increased risk of overanticoagulation. In the literature there is only limited information on the association between dietary intake of vitamin K and overanticoagulation. Two case reports described overanticoagulation after discontinuation of a weekly consumption of 750 to 1000 grams of liver [7, 8]. In a case-control study on risk factors for overanticoagulation, the habitual weekly intake of twelve vitamin K_1-rich foods was inversely associated with the risk of overanticoagulation [9].

We conducted a cohort study in a large population of community-dwelling elderly to study whether patients with a habitual dietary intake of vitamin K below the amount required for normal functioning of coagulation factors, have an increased risk of overanticoagulation.

METHODS

Setting

Data were obtained from the Rotterdam Study and from the regional outpatient anticoagulation clinic. The Rotterdam Study is a prospective population-based cohort study of neurologic, cardiovascular, locomotor, and ophthalmologic diseases in the elderly. All inhabitants of Ommoord, a suburb of the city of Rotterdam in the Netherlands, aged 55 years or over and living in the district for at least one year were invited in 1990-1993 to participate in the study. The rationale and design of this study have been described elsewhere [10]. The cohort comprises 7983 individuals who were all interviewed and investigated at baseline. The regional outpatient anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. The choice of anticoagulant (phenprocoumon or acenocoumarol) is made by the referring physician. The optimal target range of coumarin anticoagulant therapy, as recommended by the Federation of Dutch Thrombosis Centers, lies between 2.5 and 3.5 INR or between 3.0 and 4.0 INR, depending on the indication for treatment. INR measurements are

performed at a mean interval of two to three weeks, the interval being six weeks at a maximum. Dosing of the coumarin is performed by a team of specialized physicians routinely working at the anticoagulation clinic, with the aid of a computerized dosing program. All laboratory, clinical and administrative data as of 1986 are stored in computerized files. For our cohort study, the data until December 31, 1998 were used.

Cohort and outcome definition

The study cohort consisted of all participants of the Rotterdam Study of whom dietary intake data have been collected and who were treated with the coumarins acenocoumarol or phenprocoumon in the study period between the baseline visit of the Rotterdam Study (1990-1993) and December 31, 1998. The cohort included prevalent users at baseline as well as incident users during follow-up. All cohort members were followed from their baseline visit of the Rotterdam Study until the first occurrence of an INR $\geq$ 6.0, the last INR assessment during the study period, death or end of the study period, whichever came first. In case a patient had multiple treatment episodes during follow-up, all episodes in the study period were considered. The date on which an INR $\geq$ 6.0 was encountered was defined as the index date.

Exposure definition

The exposure of interest in this study was habitual dietary intake of vitamin K_1 and K_2. A semiquantitative food frequency questionnaire including 170 foods and beverages was used to assess the habitual diet of each participant as consumed during the preceding year. The questionnaire has been validated and proven suitable for use in an elderly population [11, 12]. In order to calculate the intake of vitamin K, we used data on concentrations of vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones: MK4 through MK10) as have been determined in a large variety of Dutch foods at the Department of Biochemistry and Cardiovascular Research Institute, Maastricht University. The analytical method used has been described in detail elsewhere [13]. For foods included in the food frequency questionnaire that have not been analysed, concentrations were derived from data published by others [14-19]. This was not done for vitamin K_2 because of scarcity of data in the literature.

The intake of vitamin K was expressed in µg/kg body weight per day. An intake below 1 µg/kg body weight per day was considered deficient [5, 6]. If information on weight was missing (n = 7), the intake of vitamin K was calculated by reference to the mean weight of male and female cohort members. For women an intake below 72 µg/day and for men an intake below 79 µg/day was considered deficient.

Cofactors

A person's habitual diet is only one aspect of his or her lifestyle and may be related to body mass index (BMI), smoking status and alcohol consumption. In addition, a person's habitual diet may be related to the presence of an impaired liver function, heart failure or malignancies. Since

the lifestyle factors and chronic comorbidities mentioned may interfere with anticoagulant therapy and enhance the response to coumarins [20, 21], these were considered as potential confounders. Furthermore, we considered the phase of coumarin anticoagulant therapy on the index date (initiation phase, i.e. day 1 to day 41, or stabilized phase, i.e. ≥ 42 days) and, for patients in the stabilized phase, the mean monitoring interval in the three months preceding the index date.

Statistical analysis

The cohort included prevalent users of coumarins at baseline as well as incident users during follow-up. In addition, the moment on which incident users started anticoagulant therapy differed and multiple treatment episodes were considered. Consequently, cohort members were not necessarily receiving anticoagulant therapy during the whole follow-up. Therefore, a time-dependent Cox proportional hazards regression model was used to compute the relative risk (RR) and 95% confidence interval (CI) of overanticoagulation associated with a deficient dietary intake of vitamin K. In this model, the status of a particular determinant at the index date of each case of an INR ≥ 6.0, is compared to the status of this determinant in all cohort members who are alive and at risk for the outcome. The relative risk was adjusted for age and gender. Furthermore, we adjusted for all cofactors which were univariately associated with an INR ≥ 6.0 if this caused a change in the point estimate of more than 5 percent. Population attributable risk percentages were calculated with the formula ((RR-1)/RR) * 100 * proportion of exposed cases [22].

RESULTS

The study cohort consisted of 772 patients, with a mean follow-up (± SD) of 1650 ± 764 days. Nearly 85% of the patients used acenocoumarol and the remainder phenprocoumon. As all vitamin K deficient cases used acenocoumarol, no further distinction is made between the two coumarins. During the study period, 227 patients developed an INR ≥ 6.0. The mean duration of follow-up (± SD) in the cases was 1532 ± 627 days. Baseline characteristics of the cases and the total cohort are shown in Table 1. Men and women had a more or less similar risk of an INR ≥ 6.0. Patients of 75 years and older had an increased risk. Furthermore, the risk of overanticoagulation was associated with BMI, current smoking, an impaired liver function, heart failure and malignancies. Patients with an intermediate intake of alcohol tended to have a slightly lower risk of overanticoagulation but the difference with non-use was not statistically significant.

The median daily dietary intake of vitamin K in the total cohort was 259 µg, or 3.5 µg/kg body weight (Table 2). Subdivided into vitamin K_1 and vitamin K_2, the median daily intakes were 232 µg and 26 µg, respectively. Case patients had similar median intakes. The number of patients in

Table 1. Baseline characteristics of patients with an INR ≥ 6.0 and the total cohort

Variable	Patients with an INR ≥ 6.0	Total cohort
	n = 227	n = 772
Sex		
Male	122 (54%)	386 (50%)
Female	105 (46%)	386 (50%)
Age (years, mean ± SD)	70.7 ± 6.8	69.6 ± 7.1
55-64 years	44 (19%)	195 (25%)
65-74 years	111 (49%)	378 (49%)
≥ 75 years	72 (32%)	200 (26%)
BMI		
> 25 kg/m^2	130 (58%)	499 (65%)
20-25 kg/m^2	89 (40%)	255 (33%)
< 20 kg/m^2	5 (2%)	11 (1%)
Smoking status		
Never smoker	58 (26%)	232 (30%)
Former smoker	103 (45%)	339 (44%)
Current smoker	66 (29%)	199 (26%)
Alcohol intake		
None	68 (30%)	225 (29%)
≤ 15 g/day	106 (47%)	366 (47%)
15-30 g/day	30 (13%)	107 (14%)
> 30 g/day	23 (10%)	74 (10%)
Impaired liver function*	2 (1%)	8 (1%)
Heart failure†	56 (25%)	128 (17%)
Malignancies†‡	37 (16%)	85 (11%)

* Defined as serum aminotransferases or bilirubine > 2x the upper level of normal.
† Assessed by reference to the index date.
‡ A diagnosis of a malignancy prior to the index date or within the first year after the index date.

Table 2. Daily dietary intake of vitamin K (median (interquartile range)) in the patients with an INR ≥ 6.0 and the total cohort*

Variable	Patients with an INR ≥ 6.0	Total cohort
All cohort members	n = 227	n = 772
Intake of vitamin K (µg)	254 (140)	259 (134)
Intake of vitamin K (µg/kg)	3.6 (1.9)	3.5 (1.8)
Intake of vitamin K_1 (µg)	228 (137)	232 (128)
Intake of vitamin K_2 (µg)	25 (17)	26 (19)
Vitamin K deficient patients	n = 7 (3.1%)	n = 12 (1.6%)
Intake of vitamin K (µg)	64 (37)	57 (28)
Intake of vitamin K (µg/kg)	0.71 (0.40)	0.73 (0.27)
Intake of vitamin K_1 (µg)	20 (30)	36 (30)
Intake of vitamin K_2 (µg)	15 (9)	16 (21)

* Because of the use of medians, the intake of vitamin K_1 and K_2 do not add up to the intake of vitamin K.

Table 3. The relative risk of overanticoagulation (INR ≥ 6.0) associated with a deficient dietary intake of vitamin K

Variable	RR_{crude} (95%CI)*	$RR_{adjusted}$ (95%CI)†
Deficient dietary intake of vitamin K	9.2 (4.0-20.8)	9.6 (4.0-23.0)
Sex		
Male	1.0 (reference)	1.0 (reference)
Female	1.2 (0.9-1.5)	0.8 (0.6-1.1)
Age		
55-64 years	1.0 (reference)	1.0 (reference)
65-74 years	1.0 (0.7-1.5)	1.1 (0.8-1.6)
≥ 75 years	1.5 (1.03-2.2)	1.7 (1.2-2.6)
BMI		
> 25 kg/m^2	1.0 (reference)	1.0 (reference)
20-25 kg/m^2	1.6 (1.2-2.1)	1.3 (0.99-1.8)
< 20 kg/m^2	1.2 (0.5-2.9)	1.0 (0.4-2.6)
Smoking status		
Never smoker	1.0 (reference)	1.0 (reference)
Former smoker	1.0 (0.7-1.4)	1.0 (0.7-1.6)
Current smoker	1.5 (1.02-2.1)	1.6 (1.05-2.5)
Alcohol intake		
None	1.0 (reference)	1.0 (reference)
≤ 15 g/day	0.8 (0.6-1.1)	1.2 (0.8-1.7)
15-30 g/day	0.6 (0.4-1.01)	1.0 (0.6-1.7)
> 30 g/day	0.8 (0.5-1.3)	1.0 (0.6-1.6)
Impaired liver function	6.6 (1.5-28.7)	9.7 (2.2-43.0)
Heart failure	1.6 (1.1-2.1)	1.5 (1.1-2.1)
Malignancies	2.3 (1.6-3.3)	2.6 (1.7-3.8)

* Univariate analyses of relative risks were done with the time-dependent Cox proportional hazards regression model. In this model, the status of a particular determinant at the index date of each case of an INR ≥ 6.0, is compared to the status of this determinant in all cohort members who are alive and at risk for the outcome. Hence, crude RRs cannot be calculated with the numbers in this table.
† Adjusted for sex, age, BMI, smoking status, alcohol intake and impaired liver function (missing value indicators included) at baseline, and heart failure and malignancies by reference to the index date.

the total cohort with a deficient dietary intake of vitamin K was 12 (1.6%). In these patients, the median daily intake of vitamin K was 57 µg; that of vitamin K_1 was 36 µg; and that of vitamin K_2 was 16 µg. Expressing the intake of vitamin K in µg/kg body weight per day, the intake ranged from 0.20-1.0 µg/kg body weight per day, with a median of 0.73. Of the cases, seven patients (3.1%) had a deficient dietary intake of vitamin K, with a median intake of 0.71 µg/kg body weight per day. In the vitamin K deficient cases, the median daily intakes of vitamin K_1 and vitamin K_2 were 20 µg and 15 µg, respectively. The crude relative risk of overanticoagulation associated with a deficient dietary intake of vitamin K was 9.2 (95%CI: 4.0-20.8) (Table 3). Only in one out of the seven patients with a deficient vitamin K intake, overanticoagulation occurred within the first week of treatment. After adjustment for potential confounders the relative risk was 9.6 (95%CI: 4.0-23.0). The population attributable risk percentage of overanticoagulation associated with a deficient dietary intake of vitamin K in elderly outpatients of an anticoagulation clinic was

2.8%. Adjustment for the phase of coumarin anticoagulant therapy on the index date did not substantially change the relative risk. For patients in the stabilized phase, i.e. treated for at least six weeks, the relative risk was also adjusted for the mean monitoring interval in the three months preceding the index date. The adjusted relative risk of overanticoagulation associated with a deficient dietary intake in these patients was 7.1 (95%CI: 2.7-18.6). The corresponding population attributable risk percentage was 2.4%.

DISCUSSION

In this population-based cohort study, a deficient intake of vitamin K as calculated from the total diet using data on concentrations of vitamin K_1 and vitamin K_2 in foods, was associated with a considerably increased risk of an INR $\geq$ 6.0. Apparently, when the habitual dietary intake of vitamin K is below the amount required for normal functioning of coagulation factors, regular monitoring and adjustment of the dose of coumarins does not abolish the risk of overanticoagulation. To minimize the risk of bleeding complications, patients on coumarin anticoagulant therapy should be advised to consume vitamin K-rich foods such as green, leafy vegetables.

In addition to the fact that the intake of vitamin K is insufficient for normal functioning of coagulation factors, patients on coumarins with a deficient dietary intake of vitamin K are at increased risk of overanticoagulation by antibacterial drugs that may interfere with bacterial synthesis of vitamin K in the colon [23]. The contribution of bacterial synthesis of vitamin K in the colon to the vitamin K status becomes important when the dietary intake of the vitamin is markedly decreased [24, 25]. As none of our vitamin K deficient cases was exposed to antibacterial drugs on the index date, we were not able to confirm the increased risk of overanticoagulation by antibacterial drugs in patients on coumarins with a deficient dietary intake of vitamin K. If overanticoagulation occurs within the first week of treatment, it may be caused by a relatively too high starting dose of coumarins. In our study, however, this concerned only one vitamin K deficient patient and did not explain the increased risk of overanticoagulation in patients with a deficient dietary intake of vitamin K.

Some potential limitations should be considered in the interpretation of our findings. Selection bias was probably negligible because we identified all users of coumarin anticoagulants in a defined population and because regular INR monitoring makes it unlikely that cases were missed. Information bias is also unlikely because all data on exposure and outcome were gathered prospectively and recorded similarly for all cohort members without prior knowledge of our study hypothesis. Misclassification of exposure may be present since the habitual dietary intake of vitamin K was assessed at baseline and may have changed during follow-up. However, misclassification of exposure usually leads to a conservative estimate of the relative risk. This suggests that the actual risk of overanticoagulation in patients with a deficient dietary intake

of vitamin K may be higher. Potential confounding by sex, age, BMI, smoking status, alcohol consumption, impaired liver function, heart failure, and malignancies was dealt with in the multivariate analyses. Similarly, the phase of therapy on the index date and, for patients in the stabilized phase, the mean monitoring interval in the three months preceding the index date, were considered. Although our study pertained to the coumarins acenocoumarol and phenprocoumon, it is likely that the results can be extrapolated to warfarin because here, vitamin K plays a similar role.

In our study population, and in the Netherlands in general, the dietary intake of vitamin K is high and vitamin K deficiency is rare. Therefore, the public health impact of a deficient dietary intake of vitamin K on overanticoagulation is probably modest considering the population attributable risk percentage in elderly outpatients of an anticoagulation clinic of 2.8%. However, in the USA, the mean dietary intake of vitamin K_1 is much lower and only 80 µg/day in young adults and 150 µg/day in older adults [26]. In these populations, the occurrence of an INR $\geq$ 6.0 associated with a deficient dietary intake of vitamin K, may concern many more patients.

In conclusion, in this population-based cohort study, outpatients of an anticoagulation clinic with a deficient dietary intake of vitamin K had an increased risk of overanticoagulation. Since overanticoagulation is associated with an increased risk of hemorrhages, patients on coumarin anticoagulant therapy should be advised to consume vitamin K-rich foods such as green, leafy vegetables.

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Chapter 6

General discussion

INTRODUCTION

One of the most challenging areas of research in pharmaco-epidemiology and clinical pharmacology is to understand why individuals have a different response to drug therapy.

When several patients are prescribed the same recommended daily dosage of a drug, the drug can be efficacious in most, have little or no effect in others, and/or result in adverse drug reactions (ADRs) in a small group of patients [1]. ADRs can result in significant patient morbidity, mortality, and excess medical care costs [2, 3]. A widely cited meta-analysis estimated that anually more than 2 million hospitalized patients have severe adverse drug reactions in the United States even when drugs are appropriately prescribed and administered, and that ADRs ranked between the fourth and sixth leading causes of death in the United States in 1994 [4]. Several reports and policy initiatives have urged greater efforts to reduce the rate of adverse events in medical care [3, 5-7].

Besides the importance of clinical factors that determine variability in drug response, including age, organ function, concomitant diseases, concomitant drug therapy, nutritional status, and patient compliance, it is now clear that inherited factors can have an even greater influence on the efficacy and toxicity of drugs [8, 9]. Unlike environmental factors, inherited determinants generally remain stable throughout a person's lifetime [10]. The aim of this thesis was to determine the importance of genetic variability and several environmental factors on the anticoagulant level during therapy with coumarin anticoagulants, a drug group with a narrow therapeutic index and a potentially life-threatening bleeding risk. The clinical implications of these findings lies in the possibility of prevention or early detection of excessive anticoagulation, and thus of bleeding complications, by individualization of drug therapy based on genetic and environmental information. The shortcomings and merits of the individual studies presented have been discussed in the previous chapters. In this discussion, the main findings are discussed and placed in a broader perspective.

MAIN FINDINGS

Genetic variability and the coumarin anticoagulant level

Clinical observations of inherited differences in drug response were first documented in the 1950s [11-14], giving rise to the field of pharmacogenetics. This field was initially restricted to drug metabolising enzymes. It has recently progressed to drug transporters, receptors, and other targets that can modulate drug response. This rapid extension is in close relation with the recent completion of the draft sequence of the human genome and the discovery that about 0.1% of its sequence is polymorphic. The goal of pharmacogenetics for the next years is clearly to determine the clinical consequences of the 2-3 million single nucleotide polymorphisms (SNPs), and haplotypes.

The consequences of genetic variability will depend on the extent to which the function of an encoded gene product is affected by the mutation and the frequency with which the mutation occurs. Schematically, two situations can be distinghuised [15]. First, frequent SNPs (allele frequency >10%), which have a low impact on drug response (relative risk <2). Such situations, which are by far the most frequent, have no clinical relevance for a single patient to predict the response to a particular drug. CYP3A and MDR1 allelic variants are good examples of such frequent situations. Second, rare SNPs, which dramatically alter the expression or the activity of a target protein, can sometimes have a real clinical relevance (relative risk >5), usually to predict ADRs. There are only few examples that can illustrate this rare situation, one of which is the CYP2C9 genetic polymorphism.

In chapter 2.1 we studied the effect of the SNPs CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) on the stability of the anticoagulant level during initiation and maintenance phases of acenocoumarol and phenprocoumon. In chapter 2.2 we studied the effect of these variant alleles on bleeding complications. For acenocoumarol-treated patients, the first INR after a standard starting dose tended to be higher in patients with one or more variant alleles than in wild type patients. This difference was not present anymore during the second INR measurement, probably because in the anticoagulation clinics, doses are adjusted according to the INR value obtained during the first measurement. During the rest of the initiation phase, however, patients with a variant allele on acenocoumarol appeared to be at a higher risk of overanticoagulation, but there was no effect of genotype on bleeding risk. During the maintenance phase carriers of variant alleles required significantly lower doses of acenocoumarol, and had a higher risk of major bleeding complications than wild type patients. For patients on phenprocoumon, there were no differences in these parameters between carriers of variant alleles and wild type patients.

An association between carriership of CYP2C9 allelic variants and lower warfarin dose requirements has been convincingly demonstrated [16-26] but little was known about the association with acenocoumarol and phenprocoumon. Recently, however, a study in patients receiving phenprocoumon found that both SNPs had only minor impact on the pharmacokinetics of phenprocoumon [27]. These results are in line with our study. Several recent studies have demonstrated along with our results, that carriers of a CYP2C9*3 allele also require significantly lower doses of acenocoumarol [28-32]. Whereas the CYP2C9*2 allele clearly seems to decrease the metabolism of warfarin, the effect on acenocoumarol dose requirements is suggested to be less evident. Different clinical studies demonstrated no, or a minor association between the CYP2C9*2 allele and acenocoumarol response [29-33]. We found that carriers of a CYP2C9*2 allele had a significantly higher first INR, a higher mean INR and a higher risk of overanticoagulation during the rest of the initiation phase, and had a significantly lower maintenance dose. These statistically significant associations are possibly due to the large number of patients in our study. However, because we found an increased, although not statistically significant, relative risk of a major bleeding event in carriers of a CYP2C9*2

allele, the effect of this SNP on acenocoumarol pharmacokinetics and pharmacodynamics cannot be considered clinically irrelevant. In the literature, there is lack of agreement among studies on the risk of genotype on bleeding complications of coumarin anticoagulant therapy [16, 17, 19, 21, 26, 29, 34]. The critical question is whether knowledge of an individual's CYP2C9 genotype would lead to improved optimisation of therapy, including a reduced risk of bleeding complications. Given the implied and possibly more significant involvement of environmental determinants in coumarin effects [23, 24, 32, 35-37], the answer to this question is not obvious. Phillips et al have outlined criteria that can be used to evaluate the potential impact of pharmacogenetic information in reducing ADRs [38]. The potential effect will be a function of medical need, clinical utility, and ease of use. Medical need will be driven primarily by the prevalence of variant alleles in the population, the use of a drug in that population, the severity of the ADR, and the ability to monitor drug toxicity using current technologies. Pharmacogenetic testing will be clinically appropriate only if there is sufficient evidence to link variant alleles with valid surrogate markers of drug toxicity or patient outcomes. And finally, genetic tests must be easy to use, and clinicians must be able to utilize genotype information to improve patient management and outcomes. Based on the high incidence of ADRs caused by coumarin anticoagulants, the potential effect of an intervention to reduce ADRs could be high because of the high usage of coumarin anticoagulants, the relatively high prevalence of poor metabolizers, and the severity of outcomes. For acenocoumarol [39] and warfarin [19, 21] the critical link between variant alleles and clinical outcome has now been demonstrated. The CYP2C9 enzyme genotype assays are readily performed at the clinical research level and are being developed for commercial use [9]. An argument against pharmacogenetic testing could be that coumarin anticoagulant therapy is already individualized by regular INR monitoring. However, we demonstrated for acenocoumarol that this is not enough to prevent major bleeding complications [39]. For acenocoumarol, subsequent studies are needed to determine whether clinicians are able to interpret the results and appropriately use the information and whether the additional costs of pre-dosing screening is counterbalanced by reduction of costs of treatment of bleeding complications. For phenprocoumon, we believe routine genotyping is not of additional benefit. CYP2C9 polymorphisms do not seem to play such major role in the biotransformation of this drug that knowledge of an individual's genotype would lead to improved optimisation of therapy. For phenprocoumon, a pragmatic approach in which high-risk periods are identified and managed by intensified monitoring and pre-emptive dose reductions is likely to be a more effective risk reduction strategy than pharmacogenetic testing.

In view of the extent of interindividual variability in dose requirements still observed within the various CYP2C9 genotype groups, it is possible that there are also other genetic factors involved. Vitamin K is an essential cofactor for the synthesis of several blood coagulation factors. It has been suggested that ApoE genotype has profound effects on vitamin K status. Therefore, we investigated in chapter 2.4 whether this common genetic polymorphism influenced dose

requirements and effects of acenocoumarol and phenprocoumon. Although ApoE genotype affects dose requirements of acenocoumarol, the clinical consequences of carrying a variant allele seem to be mild. Patients with a variant allele had a slightly increased INR but the risk of bleeding events seems to be negligible.

Environmental factors and the coumarin anticoagulant level

Several studies suggest that the impact of environmental factors, such as aging, drug interactions, and diet is greater than that of genetic determinants [23, 24, 32, 35-37]. A very large number of drugs have been suspected of interacting with coumarin anticoagulants [40-42]. Often, this suspicion originated from the interpretation of a single clinical event [40]. Reports of such clinical impressions are useful, but they should be considered mere leads and should be followed by epidemiological studies. In chapter 3.1 and 3.2 we investigated which antibacterial drugs and antifungal agents were associated with overanticoagulation. These drug groups are frequently mentioned as risk factors for overanticoagulation in anecdotal reports. The most powerful potentiating drugs are those that interfere with the biotransformation of the coumarin anticoagulants, such as sulfamethoxazole combined with trimethoprim, and miconazole. Some medical textbooks on drug interactions take note of the potential interaction between laxatives and coumarin anticoagulants, but epidemiological evidence that this interaction is of practical importance was lacking. Our study described in chapter 3.3 showed that the only laxative with a moderately increased risk of overanticoagulation was lactulose. In chapter 3.4, we demonstrated that there is a pharmacological interaction between nonsteroidal anti-inflammatory drugs (NSAIDs) and coumarin anticoagulants, not via platelet inhibition, but again via inhibition of the CYP2C9-mediated metabolism of coumarins. For clinical practice, it seems advisable that one should frequently monitor for INR changes when adding or deleting any drug suspected to cause an interaction with coumarin anticoagulant therapy. A clear contraindication against concomitant treatment can be proven only for some drugs.

Pathophysiological changes may also contribute to altered coumarin responsiveness. In the study described in chapter 4.1 we found that heart failure is an independent risk factor for overanticoagulation. Our data indicated that coumarin responsiveness is already slowly increasing during the weeks preceding the incident heart failure date. An extensive assessment of disease states that might increase the bleeding risk related to coumarins needs to be conducted and documented before initiating coumarin anticoagulant therapy. Doses should be adjusted downward in the presence of disease states such as, e.g. heart failure and patients should be closely monitored for signs of overanticoagulation.

The study described in chapter 5.1 showed that patients with a habitual dietary intake of vitamin K below the amount required for normal functioning of coagulation factors, i.e. 1 µg/kg body weight per day, had an increased risk of overanticoagulation. Patients should be encouraged to maintain consistency in their vitamin K intake and should strive to meet the recommended dietary allowance for vitamin K.

Gene-environment interactions

That drug response can be modified by an individual's genotype was demonstrated in two of our studies. The combination of genotype and exposure results in a higher risk of an adverse outcome than would be expected from the effect of each risk factor alone. We found that the relative risk of myocardial infarction in women for CYP2C9 genotype alone was close to unity. The relative risk for exposure to CYP2C9 substrates alone was about twofold, whereas the combined relative risk for genotype and exposure was 5.0, indicating interaction between genotype and exposure (chapter 2.3). In chapter 3.4 we demonstrated that the pharmacological interaction between NSAIDs and coumarin anticoagulants, resulting in a higher risk of overanticoagulation, was modified by CYP2C9 allelic variants.

These studies underline that drug response is complex, resulting not only from underlying genotypic variability and variability in environmental factors, but also from interactions between these factors.

Unknown genetic and environmental determinants

A few recently published studies suggest that environmental factors such as age, drug interactions, and diet do not fully account for the non-CYP2C9-associated variability in coumarin dose requirements [23, 24, 32]. This strongly suggests that other, currently unknown environmental determinants or possibly unidentified genetic variants may be involved, especially in the 5'-flanking region or the transcriptional regulatory receptors of the gene [43]. In addition, the possibility of genetically determined variability in the various proteins involved in the anticoagulant effect, which may contribute to differences in coumarin dose requirements, is a largely unexplored area with regard to interindividual variability in coumarin responsiveness [44].

Acenocoumarol versus phenprocoumon

Our results suggest less impact of the CYP2C9 polymorphisms on dose requirements and anticoagulation stability of phenprocoumon than of acenocoumarol. Thus, if the variability in biotransformation is a major cause of the overall incidence of ADRs during use of coumarin anticoagulants, the incidence of ADRs should be lower during treatment with phenprocoumon. Indeed, we found lower relative risks for all, and for minor bleeding complications in phenprocoumon-treated patients. But it should be noted that a direct comparison between acenocoumarol and phenprocoumon was not performed, and that the number of patients in our studies was limited. Our findings confirm previous observations that longer-acting coumarins provide a higher quality of therapy than short-acting coumarins [45-47].

METHODOLOGICAL CONSIDERATIONS

The studies in this thesis raised a number of interesting methodological considerations.

SNP analysis versus haplotype analysis

While it was often brought forward during the last decade that SNPs could predict particular clinical outcomes, it is now proposed that SNPs alone are not good predictors of a drug response and that haplotype analysis should replace SNP analysis [48]. A haplotype is a group of alleles found at linked loci on a single chromosome, usually inherited as a unit. Haplotype analysis implicates the simultaneous detection of numerous SNPs on the same gene and, therefore, increases the cost for its determination. It should be kept in mind that haplotype analysis is an epidemiologic strategy that should be extrapolated to individual risk prediction with great caution; most of the time, individual haplotypes are statistical estimations and are not observed, unless the subjects are heterozygous at one SNP at most [32]. Furthermore, to limit the number of polymorphic markers ("tagging SNPs") needed to construct and determine major haplotypes, minor haplotypes representing a few percent of the studied population will be excluded. This might probably represent an important bias because rare clinical outcomes are usually associated with rare genetic variants, which will be missed with usual haplotype analysis [15]. The predictive value of individual coding SNPs and haplotypes (combinations of the 5'-flanking and coding SNPs), were recently compared [15, 32]. The haplotype which included the CYP2C9*3 SNP, was associated with a more profound response to coumarin anticoagulants. A similar conclusion, however, could be obtained when the CYP2C9*3 SNP was taken alone in the analysis (SNP analysis). Haplotypes which include the CYP2C9*2 SNP also do not seem to predict more genetic variability than this coding SNP alone [32, 49]. This means that most of the information on the genetic variability of CYP2C9 is related to two SNPs and haplotype analysis does not seem to provide additional information.

Adequate sample size gene-environment interaction

Efforts to study gene-environment interactions are tempered by the difficulty in obtaining adequate sample size [50-52]. In both studies on gene-environment interaction (chapter 2.3 and 3.4) we therefore classified drug exposure and genotype as being either present or absent. An advantage of these dichotomized data is that it gives us direct insights into the relative risk estimates for each factor alone and their joint effect. Unfortunately, this reduction into two categories results in general in a loss of information by misclassification and is a simplification of the complexity of biology.

Disease misclassification

In several studies we used overanticoagulation as outcome variable. When two subsequent INR measurements had a value below 6.0 we assumed that in between the INR value was

also below 6.0. Because patients in an anticoagulation clinic are regularly assessed for their INR it seems unlikely that in this way many cases of overanticoagulation were missed. When the proportion of subjects misclassified on disease does depend on exposure differential misclassification occurs, which can either exaggerate or underestimate an effect. This may play a role in the associations of several drug interactions with overanticoagulation, since patients are instructed to inform the clinic of these occurrences. If considered necessary, the patient's INR is measured earlier than the appointed date. Then, the chance of diagnosing the outcome is different for exposed and unexposed individuals. We dealt with this bias by adjustment for earlier INR-assessment.

Repeated measurement analysis

With the development of statistical techniques, it has become possible to analyse longitudinal relationships using all available longitudinal data, without summarizing the longitudinal development of each subject into one value. Because with these sophisticated statistical techniques the outcome variable is repeatedly measured on the same subject, these methods adjust for the dependency of observations within one subject. In the study on the effect of CYP2C9 variant alleles on the coumarin maintenance dose we simply calculated the mean maintenance dose as outcome variable (chapter 2.1). We found the longitudinal methods to be much more efficient in detecting a difference in outcome for the different exposure groups in our study on the effect of ApoE genotype on the coumarin maintenance dose (chapter 2.4). The magnitude of the differences we found were the same with the longitudinal method as by calculating a mean dose per subject (data not shown), but the statistical significance of the differences with the wild type genotype was much larger with the longitudinal method.

FUTURE RESEARCH

In view of the extent of interindividual variability in coumarin dose requirements still observed within the various CYP2C9 genotype groups, it is possible that genotyping for additional polymorphic genes could be of value. Further studies on this aspect may provide useful information. Use of coumarin anticoagulants is still increasing but the development of novel oral anticoagulants, such as the direct thrombin inhibitors which do not require coagulation monitoring and which plasma concentrations are directly proportional to dose, may lead to a decrease of coumarin use in the future. Clinical trials comparing oral direct thrombin inhibitors and coumarins are underway [53]. A direct cost-benefit comparison of direct thrombin inhibitors with the older and cheaper coumarin anticoagulants combined with CYP2C9 genotyping under everyday circumstances would be interesting. As long as we miss such data, coumarins may remain useful in the treatment of thromboembolic disorders.

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Chapter 7

Summary

Chapter 7.1

Summary

Coumarin anticoagulants are drugs with a low therapeutic index. Coumarins have a large pharmacokinetic and pharmacodynamic interindividual variability, and may cause life-threatening bleeding complications. Most of the extensive research on coumarin anticoagulant therapy has focussed on warfarin. Because of the different pharmacokinetic properties of each individual drug, the results of these studies can probably not be directly extrapolated to the other coumarin anticoagulants. Therefore, the aim of this thesis was to study the various genetic and environmental factors affecting the anticoagulation levels of acenocoumarol or phenprocoumon among outpatients of an anticoagulation clinic. The studies described in this thesis have been performed in the Rotterdam Study, a prospective population-based cohort study, which was initiated to assess prevalence, incidence, and determinants of diseases in the elderly.

Chapter 1 gives a general introduction to coumarin anticoagulant therapy.

In **chapter 2**, studies on the association between several polymorphisms and dose requirements and clinical effects are presented. In **chapter 2.1**, we investigated the cytochrome P450 polymorphisms, CYP2C9*2 and CYP2C9*3, in relation to the international normalised ratio (INR) during the first six weeks of treatment and its effect on the maintenance dose in a cohort of 1124 patients from the Rotterdam Study who were treated with acenocoumarol or phenprocoumon between January 1, 1985 and December 31, 1998. There was a statistically significant difference in first INR between patients with variant genotypes and those with the wild type. Almost all acenocoumarol-treated patients with a variant genotype had a significantly higher mean INR and had a higher risk of an INR $\geq$ 6.0 during the first six weeks of treatment. A clear genotype-dose relationship was found for acenocoumarol-treated patients. For patients on phenprocoumon, no significant differences were observed between variant genotypes and the wild type genotype. In **chapter 2.2**, we examined the effect of the CYP2C9 polymorphisms on bleeding complications during initiation and maintenance phases of coumarin anticoagulant therapy. The design was a population-based cohort study in 996 patients on acenocoumarol or phenprocoumon, for whom INR data were available and whose CYP2C9 status was known. For patients with the wild type genotype, the rate of minor bleeding, major bleeding and fatal bleeding was 15.9, 3.4 and 0.2 per 100 treatment years, respectively. For patients with a variant allele, the rate of minor, major and fatal bleeding was 14.6, 5.4 and 0.5 per 100 treatment years. Patients with a variant allele on acenocoumarol had a significantly increased risk of a major bleeding event (relative risk (RR) 1.83, 95%CI: 1.01-3.32). We did not find such an association for phenprocoumon. During the initiation phase of therapy we found no effect of variant genotype on bleeding risk. That CYP2C9 allelic variants not only affect variability in drug response but are also involved in the metabolism of endogenous substances, is demonstrated in **chapter 2.3**. We investigated whether CYP2C9 allelic variants and use of CYP2C9 substrates were associated with an increased risk of myocardial infarction in 2210 men and 3534 women from the Rotterdam Study. In women, use of CYP2C9 substrates was significantly associated with myocardial infarction (RR 2.68, 95%CI: 1.60-4.49). The risk of myocardial infarction was fivefold in female users of CYP2C9 substrates with a variant allele.

Neither the use of CYP2C9 substrates, nor the variant alleles were associated with an increased risk of myocardial infarction in men. The influence of the apolipoprotein E polymorphism on coumarin dose requirements, overanticoagulation and bleeding complications are discussed in **chapter 2.4**. In a cohort study among 1637 patients on acenocoumarol or phenprocoumon, we found that individuals homozygous for the ε4 allele required a significantly lower dose of acenocoumarol than patients with genotype ε3/ε3 to attain the same level of anticoagulation. Patients homozygous for the ε2 allele on acenocoumarol required a significantly higher dose than patients with genotype ε3/ε3. The effect of the ε4 allele on the coumarin dose was dose-dependent, while the effect of the ε2 allele was not. No significant dose differences were observed for phenprocoumon.

Chapter 3 focuses on the role of drug interactions in overanticoagulation. **Chapters 3.1**, **3.2**, **3.3** and **3.4** are based on the same population-based cohort study in a sample of the Rotterdam Study. The study cohort consisted of all participants who were treated with acenocoumarol or phenprocoumon in the study period from April 1, 1991 through December 31, 1998 (chapter 3.4 through May 31, 2003) and for whom INR-data were available. All cohort members were followed until the first occurrence of an INR $\geq$ 6.0, the last INR-assessment because of the end of their treatment, death or end of the study period. For data on comedication, we used data from regional pharmacies where more than 99% of participants fill their prescriptions. In **chapter 3.1** we describe which antibacterial drugs are associated with overanticoagulation. Of the 1124 patients in the cohort, 351 developed an INR $\geq$ 6.0. Eight antibacterial drugs were multivariately associated with overanticoagulation. Sulfamethoxazole combined with trimethoprim most strongly increased the risk of overanticoagulation (RR 20.1; 95%CI 10.7-37.9). Stratification showed that the induction period of overanticoagulation varied between different antibacterial drugs. In **chapter 3.2** we demonstrated that some antifungal agents were associated with a strongly increased risk of overanticoagulation. The relative risk varied considerably between the different agents and the different routes of administration. The strongest risk increase was associated with oral miconazole (RR 36.3; 95%CI: 12.4-108.0). The study described in **chapter 3.3** showed that a potential interaction between laxatives and coumarins is of practical importance only for lactulose (RR 3.4; 95%CI: 2.2-5.3). **Chapter 3.4** demonstrates that there is a pharmacological interaction between nonsteroidal anti-inflammatory drugs (NSAIDs) and coumarins and that this interaction is modified by allelic variants of CYP2C9. Apparently, NSAIDs may not only induce bleeding by inhibition of platelet function but also via a pharmacokinetic interaction with coumarins. Awareness of these drug interactions and more frequent monitoring of INR-values during the initial stages of concomitant drug therapy are warranted to minimise the risk of bleeding complications. The study described in chapter 3.4 underlines that drug response is complex, resulting not only from underlying genotypic variability and variability in environmental factors, but also from interactions between these factors.

Several chronic diseases are associated with overanticoagulation. Heart failure has been identified as a risk factor for increased coumarin responsiveness in several small-scale

experiments. Therefore, we investigated whether patients with heart failure have an increased risk of overanticoagulation and determined the effect of incident heart failure on coumarin dose requirements (**chapter 4**). The study cohort consisted of all patients treated with acenocoumarol or phenprocoumon between the baseline visit of the Rotterdam Study through January 1, 2000. All cohort members were followed until the first occurrence of an INR ≥ 6.0, the last INR-assessment, death, loss to follow-up, or end of the study period. Of the 1077 patients in the cohort, 396 developed an INR ≥ 6.0. The risk of overanticoagulation was 1.66 (95%CI: 1.33-2.07) for cases of prevalent heart failure and 1.91 (95%CI: 1.31-2.79) for incident cases. Given the high prevalence of heart failure, a 66% risk increase is substantial. The decrease in dose requirements in patients with incident heart failure showed a significant trend between the 5th INR measurement preceding the incident heart failure date and the 3rd measurement after this date. Patients with heart failure should be closely monitored to prevent potential bleeding complications.

In **chapter 5** we examined whether patients with a deficient dietary intake of vitamin K have an increased risk of overanticoagulation. The study cohort consisted of all participants of whom dietary intake data have been collected and who were treated with coumarin anticoagulants in the study period from the baseline visit of the Rotterdam Study (1990-1993) through December 31, 1998. All cohort members were followed until the first occurrence of an INR ≥ 6.0, the last INR-assessment during the study period, death or end of the study period. The intake of vitamin K was calculated from the total diet using data on concentrations of vitamin K_1 and vitamin K_2 in foods. An intake of vitamin K below 1 µg/kg body weight per day was considered deficient. Of the 772 patients in the cohort, 227 developed an INR ≥ 6.0 during the study period. The number of patients in the total cohort with a deficient dietary intake of vitamin K was 12 (1.6%). Of the cases, seven patients (3.1%) had a deficient dietary intake of vitamin K. The adjusted relative risk of overanticoagulation associated with a deficient dietary intake of vitamin K was 9.6 (95%CI: 4.0-23.0). To minimise the risk of bleeding complications, patients on coumarin anticoagulant therapy may be advised to consume vitamin K-rich foods such as green, leafy vegetables.

In the general discussion in **chapter 6**, the main findings are summarised and some methodological issues are discussed. In addition, the implications for coumarin anticoagulant therapy and recommendations for future research are given.

Chapter 7.2

Samenvatting

Cumarine anticoagulantia hebben een smalle therapeutische breedte en vertonen een grote interindividuele variabiliteit in farmacokinetische en farmacodynamische eigenschappen. Deze geneesmiddelen kunnen levensbedreigende bloedingen veroorzaken. Het merendeel van de vele onderzoeken op het gebied van orale antistollingsbehandeling heeft zich geconcentreerd op warfarine. Vanwege de verschillende farmacokinetische eigenschappen van de individuele stoffen, kunnen deze resultaten waarschijnlijk niet rechtstreeks worden geëxtrapoleerd naar de andere cumarine anticoagulantia. Het doel van dit proefschrift was daarom om een aantal genetische en omgevingsfactoren te bestuderen die de intensiteit van de antistollingsbehandeling met acenocoumarol of fenprocoumon, bij patiënten van een trombosedienst, beïnvloeden. Alle studies die hier worden gepresenteerd zijn uitgevoerd binnen het Rotterdamse ERGO-onderzoek (Erasmus Rotterdam Gezondheid en Ouderen), internationaal bekend als "the Rotterdam Study". Dit is een prospectief bevolkingsonderzoek naar frequentie en oorzaken van chronische ziekten bij ouderen.

Na een algemene introductie over cumarine anticoagulantia in **hoofdstuk 1**, wordt in **hoofdstuk 2** de invloed van verschillende polymorfismen op de cumarine dosering en op een aantal klinische uitkomsten gepresenteerd. In **hoofdstuk 2.1** onderzochten we de invloed van cytochroom P450 2C9 polymorfismen, CYP2C9*2 en CYP2C9*3, op de international normalised ratio (INR, maat voor de intensiteit van de antistolling) gedurende de eerste zes behandelingsweken (initiatiefase) en op de onderhoudsdosering in een cohort van 1124 patiënten die werden behandeld met acenocoumarol of fenprocoumon tussen 1 januari 1985 en 31 december 1998. Er bleek een statistisch significant verschil te bestaan in de hoogte van de eerste INR na een standaard startdosis tussen dragers van een variant allel en mensen zonder deze allelen. Patiënten die acenocoumarol gebruikten, hadden gemiddeld een hogere INR en meer kans op doorgeschoten antistolling (INR $\geq$ 6.0) gedurende de initiatiefase. We vonden een duidelijke genotype-dosis relatie voor patiënten die acenocoumarol gebruikten. Voor patiënten, die fenprocoumon gebruikten, konden we geen verschillen aantonen tussen dragers van een variant allel en mensen zonder deze allelen. In **hoofdstuk 2.2** bestudeerden we het effect van de CYP2C9 polymorfismen op het krijgen van bloedingen gedurende de initiatie- en onderhoudsfase van de antistollingsbehandeling. We deden een cohort-onderzoek onder 996 patiënten die werden behandeld met acenocoumarol of fenprocoumon, van wie INR gegevens beschikbaar waren en van wie de CYP2C9 status bekend was. De risico's op een kleine bloeding, een ernstige bloeding en dodelijke bloedingcomplicaties waren respectievelijk 15.9, 3.4 en 0.2 per 100 jaren antistollingsbehandeling voor patiënten zonder variant allelen. Voor patiënten met een variant allel waren deze risico's 14.6, 5.4 en 0.5 per 100 behandelingsjaren. Dragers van een variant allel - die acenocoumarol gebruikten - hadden een significant hoger risico op het krijgen van een ernstige bloeding (relatief risico (RR) 1.83, 95%CI: 1.01-3.32). Voor fenprocoumon vonden we deze associatie niet. Gedurende de initiatiefase bleek er geen effect te zijn van het genotype op het bloedingsrisico. Dat het CYP2C9 genotype niet alleen een belangrijke rol speelt bij het metabolisme van geneesmiddelen maar ook bij dat van endogene

stoffen werd aannemelijk gemaakt in **hoofdstuk 2.3**. We onderzochten of dragerschap van een CYP2C9 variant allel en gebruik van geneesmiddelen die via CYP2C9 worden gemetaboliseerd, geassocieerd was met het krijgen van een hartinfarct bij 2210 mannen en 3534 vrouwen in het ERGO-onderzoek. Gebruik van CYP2C9 substraten was bij vrouwen geassocieerd met het krijgen van een hartinfarct (RR 2.68, 95%CI: 1.60-4.49). Het relatieve risico op een hartinfarct was 5 maal zo hoog bij vrouwelijke dragers van een variant allel die eveneens CYP2C9 substraten gebruikten ten opzichte van vrouwen zonder variant allel die deze geneesmiddelen niet gebruikten. Bij mannen werd geen associatie gevonden. De invloed van het apolipoproteine E polymorfisme op de cumarine dosering, en het optreden van doorgeschoten antistolling en bloedingcomplicaties wordt in **hoofdstuk 2.4** besproken. In een cohort-onderzoek onder 1637 patiënten die acenocoumarol of fenprocoumon gebruikten, toonden we aan dat mensen die homozygoot zijn voor het ε4 allel een significant lagere dosis acenocoumarol nodig hebben dan patiënten met genotype ε3/ε3. Patiënten die behandeld worden met acenocoumarol en homozygoot zijn voor het ε2 allel hebben een significant hogere dosis acenocoumarol nodig dan patiënten met genotype ε3/ε3. Het effect van het ε4 allel bleek dosis-afhankelijk te zijn, dat van het ε2 allel niet. Voor patiënten, die fenprocoumon gebruikten, werden geen significante verschillen in dosering aangetoond tussen de verschillende genotypen.

Hoofdstuk 3 gaat over de rol van geneesmiddelinteracties bij doorgeschoten antistolling. De **hoofdstukken 3.1**, **3.2**, **3.3** and **3.4** zijn gebaseerd op dezelfde prospectieve cohort-analyse bij een deel van de ERGO-deelnemers. Het studiecohort bestond uit alle deelnemers die werden behandeld met acenocoumarol of fenprocoumon in de studieperiode van 1 april 1991 tot 31 december 1998 (voor de studie in hoofdstuk 3.4 tot 31 mei 2003). Alle cohortleden werden gevolgd tot het eerste optreden van een INR $\geq$ 6.0, de laatste INR-bepaling, overlijden of einde van de studieperiode. Gegevens over geneesmiddelengebruik waren afkomstig van lokale apotheken, bij wie meer dan 99% van de deelnemers de geneesmiddelen betrekt. In **hoofdstuk 3.1** onderzochten we welke antibiotica zijn geassocieerd met doorgeschoten antistolling. Van de 1124 patiënten in het cohort ontwikkelden er 351 een INR $\geq$ 6.0. Acht antibiotica waren geassocieerd met doorgeschoten antistolling. Sulfamethoxazole in combinatie met trimethoprim (co-trimoxazol) verhoogde het risico op doorschieten het sterkst (RR 20.1; 95%CI: 10.7-37.9). Stratificatie liet zien dat de periode tussen het starten van de behandeling met antibiotica en het doorschieten varieerde tussen de verschillende antibiotica.

In **hoofdstuk 3.2** toonden we aan dat enkele antischimmel-middelen waren geassocieerd met een sterk verhoogd risico op doorgeschoten antistolling. Het relatieve risico varieerde sterk tussen de verschillende middelen en de verschillende toedieningsroutes. Het risico op doorschieten was het meest verhoogd bij miconazol per os (RR 36.3; 95%CI: 12.4-108.0). De studie die in **hoofdstuk 3.3** wordt beschreven laat zien dat de theoretische interactie tussen laxantia en cumarines alleen van praktisch belang is voor lactulose (RR 3.4; 95%CI: 2.2-5.3). **Hoofdstuk 3.4** laat zien dat er een farmacokinetische interactie is tussen nonsteroidal anti-inflammatory drugs (NSAIDs) and cumarines en dat het effect van deze interactie anders is

voor dragers van een CYP2C9 variant allel. Het zich bewust zijn van deze geneesmiddel-interacties en het frequenter controleren van de INR in de beginfase van gelijktijdig gebruikte geneesmiddelen wordt aangeraden om het risico op bloedingscomplicaties te beperken. De studie beschreven in hoofdstuk 3.4 onderstreept nog eens dat geneesmiddelenrespons een complex fenomeen is, niet alleen ten gevolge van genetische verschillen en verschillen in omgevingsfactoren, maar ook ten gevolge van interacties tussen deze factoren.

Verschillende chronische ziekten worden geassocieerd met een doorgeschoten antistoll ingsbehandeling. In experimenten met een beperkt aantal patiënten in de jaren '50 van de vorige eeuw werd gevonden dat patiënten met hartfalen een grotere respons op cumarine anticoagulantia vertoonden. Wij bevestigden deze associatie in een cohort-onderzoek onder alle patiënten die acenocoumarol of fenprocoumon gebruikten in de studieperiode tussen de aanvang van het ERGO-onderzoek (1990-1993) en 1 januari 2000 (**hoofdstuk 4**). Alle cohortleden werden gevolgd tot het eerste optreden van een INR ≥ 6.0, de laatste INR-bepaling, overlijden of einde van de studieperiode. Van de 1077 patiënten in het cohort ontwikkelden er 396 een INR ≥ 6.0. Het relatieve risico op doorgeschoten antistolling geassocieerd met prevalent hartfalen was 1.66 (95%CI: 1.33-2.07) en met incident hartfalen 1.91 (95%CI: 1.31-2.79). Gezien de hoge prevalentie van hartfalen, kan een risicotoename van 66% als substantieel worden beschouwd. De afname in cumarine dosering om dezelfde intensiteit van antistolling te behouden, zet in op de 5e INR-bepaling voorafgaand aan de incidente datum van het hartfalen en duurt voort tot de 3e INR-bepaling na deze datum. De INR van patiënten met hartfalen moet frequenter worden gecontroleerd om doorschieten van de antistolling en potentiële bloedingcomplicaties te voorkomen.

In **hoofdstuk 5** onderzochten we of patiënten met een deficiënte voedingsinneming van vitamine K een verhoogd risico op het doorschieten van de antistolling hadden. We voerden hiertoe een cohort-onderzoek uit bij een deel van de ERGO-deelnemers. Het studiecohort bestond uit alle deelnemers van wie voedingsgegevens waren verzameld en die werden behandeld met cumarine anticoagulantia in de studieperiode tussen de aanvang van het ERGO-onderzoek en 31 december 1998. Alle cohortleden werden gevolgd tot het eerste optreden van een INR ≥ 6.0, de laatste INR-bepaling, overlijden of einde van de studieperiode. De inneming van vitamine K werd berekend over de totale voeding op basis van de vitamine K_1- en K_2-gehalten van voedingsmiddelen. Een vitamine K-inneming beneden 1 µg/kg lichaamsgewicht per dag werd als deficiënt beschouwd. Van de 772 patiënten in het cohort ontwikkelden er 227 een INR ≥ 6.0. Het aantal patiënten in het totale cohort met een deficiënte voedingsinneming van vitamine K was 12 (1.6%). Van de patiënten met een doorgeschoten antistolling hadden er zeven (3.1%) een deficiënte inneming van vitamine K. Het gecorrigeerde relatieve risico op doorgeschoten antistolling geassocieerd met deficiënte voedingsinneming van vitamine K was 9.6 (95%CI: 4.0-23.0). Teneinde het risico van bloedingcomplicaties zo klein mogelijk te houden zou patiënten op cumarine antistollingsbehandeling kunnen worden geadviseerd om vitamine K-rijke voedingsmiddelen te eten, zoals groene bladgroenten.

In de algemene discussie in **hoofdstuk 6** worden de belangrijkste resultaten samengevat en bespreken we een aantal methodologische aspecten. Vervolgens worden de consequenties voor de antistollingsbehandeling met cumarine anticoagulantia besproken en worden aanbevelingen gedaan voor verder onderzoek.

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Loes Visser was born on May 19, 1968 in Leeuwarden, the Netherlands. In 1986, she completed secondary school at the 'Rijksscholengemeenschap Leeuwarden'. In that same year she started her pharmacy study at the University of Groningen. After obtaining her Master of Science degree *cum laude* in 1992, she performed a research project on gender differences in drug use at the Department of Pharmacoepidemiology and Pharmacotherapy of the Utrecht Institute for Pharmaceutical Sciences. In 1995, she obtained her pharmacist's degree at the University of Groningen. Hereafter, she worked several months in the hospital pharmacy of St Jansdal hospital (head: Drs. F.M.P. Lindelauf) in Harderwijk, and at the Department of Clinical Pharmacy of the University Medical Centre, Nijmegen (head: Prof.dr. Y.A. Hekster). She did her specialist training in hospital pharmacy at the 'Apotheek Haagse ziekenhuizen' (head: Dr. I.C. Dijkhuis and subsequently, Drs. B.H. Graatsma) between 1996 and 1999. In January 2000, she started working as a hospital pharmacist in the Erasmus Medical Center, Rotterdam (head Dr.P.J. Roos). In the same period she started working on this thesis at the Department of Epidemiology & Biostatistics (head: Prof.dr. A. Hofman) of the Erasmus Medical Center, Rotterdam for two days per week. During this period she obtained a Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences. In 2003, she received an award for a part of this thesis as the most original research project from the Dutch Society of Hospital Pharmacists (NVZA). She is married to Jeroen Wakkerman. They have two daughters: Famke and Fleur.