Pharmacogenetic aspects and drug interactions in anticoagulation therapy with coumarins

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Pharmacogenetic Aspects and Drug Interactions in Anticoagulation Therapy with Coumarins

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Alles heeft zijn tijd

Voor alles wat gebeurt is er een uur,
een tijd voor alles wat er is onder de hemel.
Er is een tijd om te baren
en een tijd om te sterven,
een tijd om te planten
en een tijd om te rooien.
Er is een tijd om te doden
en een tijd om te helen,
een tijd om af te breken
en een tijd om op te bouwen.
Er is een tijd om te huilen
en een tijd om te lachen,
een tijd om te rouwen
en een tijd om te dansen.
Er is een tijd om te ontvlammen
en een tijd om te verkillen,
een tijd om te omhelzen
en een tijd om af te weren.
Er is een tijd om te zoeken
en een tijd om te verliezen,
een tijd om te bewaren
en een tijd om weg te gooien.
Er is een tijd om te scheuren
en een tijd om te herstellen,
een tijd om te zwijgen
en een tijd om te spreken.
Er is een tijd om lief te hebben
en een tijd om te haten.
Er is een tijd voor oorlog
en er is een tijd voor vrede.

Prediker 2;3: 1-9

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Chapter 1

General introduction

Introduction

The mysterious ability of blood to clot has fascinated people over millennia. In the beginning of the 20th century, the mechanisms became better understood. In 1922, the development of hemorrhagic disease in cattle caused by mouldy sweet clover was described by Schofield.[1] The absence or delay of blood clotting was correlated to a greatly diminished quantity of prothrombin. This discovery remained unnoticed until twenty years later the 'hemorrhagic agent' was identified as dicoumarol, 3,3-methylene-bis.[2, 3] It was promptly made available for clinical studies and already one year later first experiences on the effectiveness in deep vein thrombosis as well as its hemorrhagic complications were published.[2, 4, 5] From the coumarin derivates synthesized, the most potent one was warfarin, an acronym for the Wisconsin Alumni Research Foundation (WARF). As it was used successfully to fight rats, its name may also contribute to that 'warfare'. It was patented in 1948 and is since then the most frequently used coumarin worldwide. In 1929, Dam observed haemorrhage and markedly prolonged coagulation times in chickens fed with diets from which fat was completely extracted.[6, 7] He concluded that the substance whose absence in the diet was responsible for the coagulation and bone growth pathologies should be a new fat-soluble vitamin which he named vitamin K ("Koagulation"). For the discovery of vitamin K and the purification, characterization and synthesis of the vitamin, Dam and Doisy were awarded the Nobel Prize in Medicine in 1943. It was known then empirically that vitamin K reversed the bleeding problem of mouldy sweet clover poisoning. However, it took three more decades until the vitamin K cycle was proposed in 1974.[8] After another three decades, in 2004, the complex biochemical relationship between vitamin K, its epoxide, and coumarins was enlightened by identifying the *VKORC1* gene. [9, 10] This gene encodes the protein which is the target of the coumarins. Genetically mutated variants of the gene have been shown to cause warfarin-resistance phenotypes as well as pathogenic deficiency of all vitamin K-dependent coagulation factors.

Since 60 years, oral anticoagulants of the coumarin type are extensively used worldwide as the first choice in treatment and prevention of arterial or venous thrombosis.[11] Being orally administered, they have advantages over heparin, an injectable biological extract, and the thorough experience with longterm treatment of millions of patients makes them still superior to the recently available direct thrombin inhibitors. However, coumarins are difficult to handle in clinical practice as they have a narrow therapeutic range. This means that their effective dose is very close to the poisoning dose at which the risk of disabling or lethal major bleeding occurs, the most feared complication of coumarin treatment. Furthermore, dosage needs of coumarins can differ between persons tenfold and change also intra-individually over time. Currently, bleeding by coumarins is one of the most frequent iatrogenic causes of hospital admission. It might be preventable by better insight into individual drug response. [12, 13] It is an ongoing matter of concern to find the right balance between benefits and

risks of coumarin treatment. The aim of this thesis is to contribute to the ongoing research to assess relevant risk factors for blood coagulation, to predict individual risks in anticoagulation therapy and to develop strategies for improvement of medication safety.

Blood coagulation cascade

Blood coagulation depends on a cascade of reactions between various factors (figure 1)[14]. It starts after direct contact of the tissue factor with blood, which leads to formation of the TF-factor VIIa-complex. This activates factor X, that stimulates production of thrombin and finally the change of fibrinogen to fibrin. The fibrin network surrounds the clot of thrombocytes and forms the thrombus, together with erythrocytes and leucocytes. Obstruction of a blood vessel by a thrombus can result into thrombosis or embolism. Arterial thrombosis and embolism can lead to myocardial infarction or stroke. Venous obstructions encourage development of deep venous thrombosis or pulmonary embolisms. As venous thrombi consist primarily of fibrin and erythrocytes they can be treated effectively with anticoagulants. Arterial thrombi are composed mostly of thrombocytes and their treatment requires combinations of anticoagulants, thrombotic aggregation inhibitors and thrombolytic drugs.[15]

Coumarin treatment and pharmacodynamics

The effectiveness of coumarins has been established by well-designed clinical trials for the primary and secondary prevention of venous thromboembolism, for the prevention of systemic embolism in patients with prosthetic heart valves or arterial fibrillation, for the primary prevention of acute myocardial infarction in high-risk men, and for the prevention of stroke, recurrent infarction, or death in patients with acute myocardial infarction.[15] Therapy with other drugs used for prevention or treatment of thromboembolic diseases such as heparins, thrombolytic and platelet inhibiting drugs such as aspirin, and glycoprotein IIb/IIIa antagonists and thrombin inhibitors such as dabigatran, are beyond the scope of this thesis and will not be discussed further.

Coumarins are antagonists of vitamin K, a fat-soluble vitamin that is essential for the formation of the hepatic coagulation factors II (prothrombin), VII, IX, and X, as well as protein C, S and Z, and Matrix Gla protein (MGP).[6, 16] These clotting factors and proteins are glycoproteins with glutamic acid residues (Glu), which are transformed by γ-carboxylation into γ-carboxyglutamic (Gla) residues.[17] Calcium binding of the Gla residues of the coagulation factors leads to the conformational changes that are needed for their effects in the coagulation cascade.[18]

Figure 1 Blood coagulation[58]

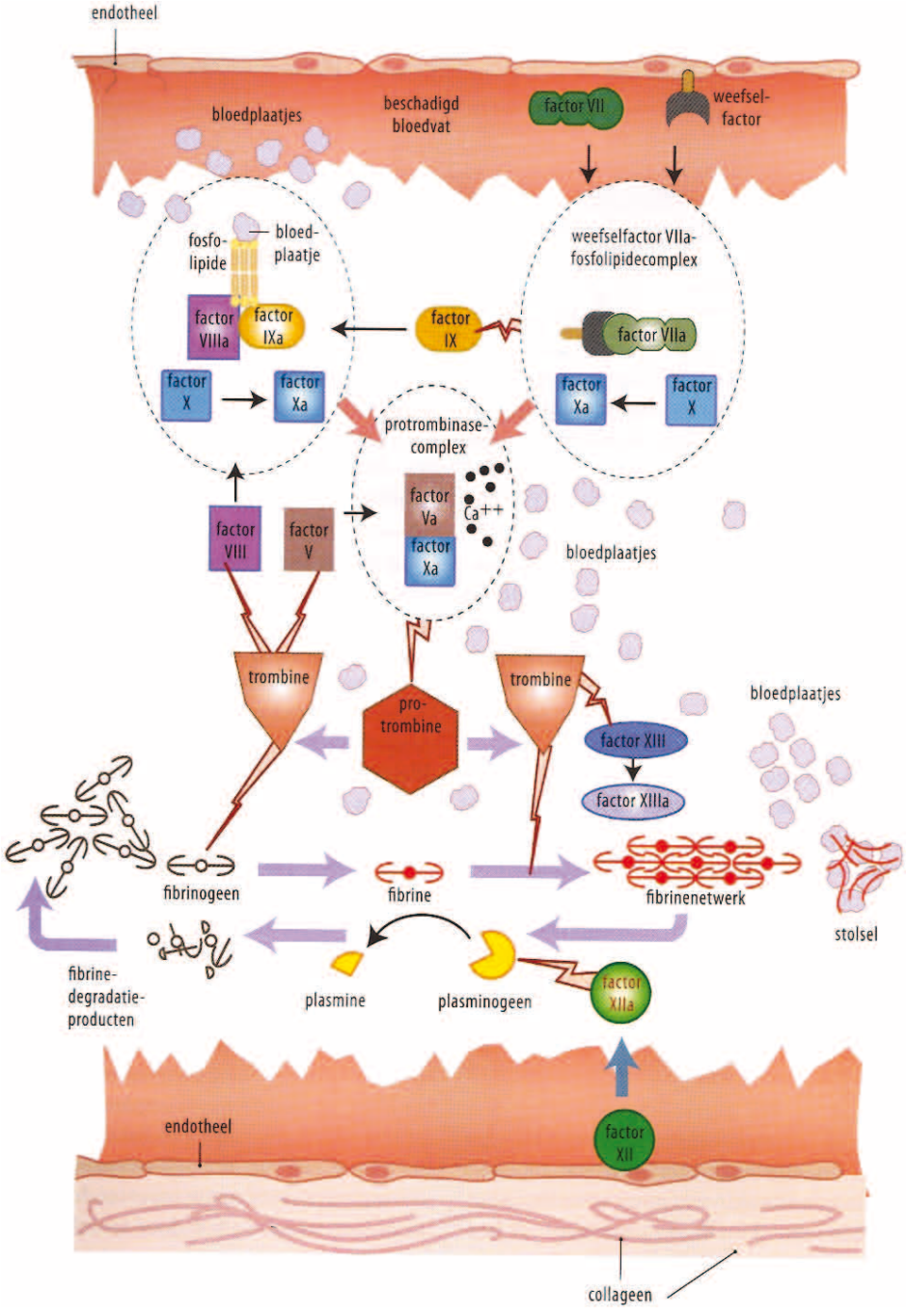
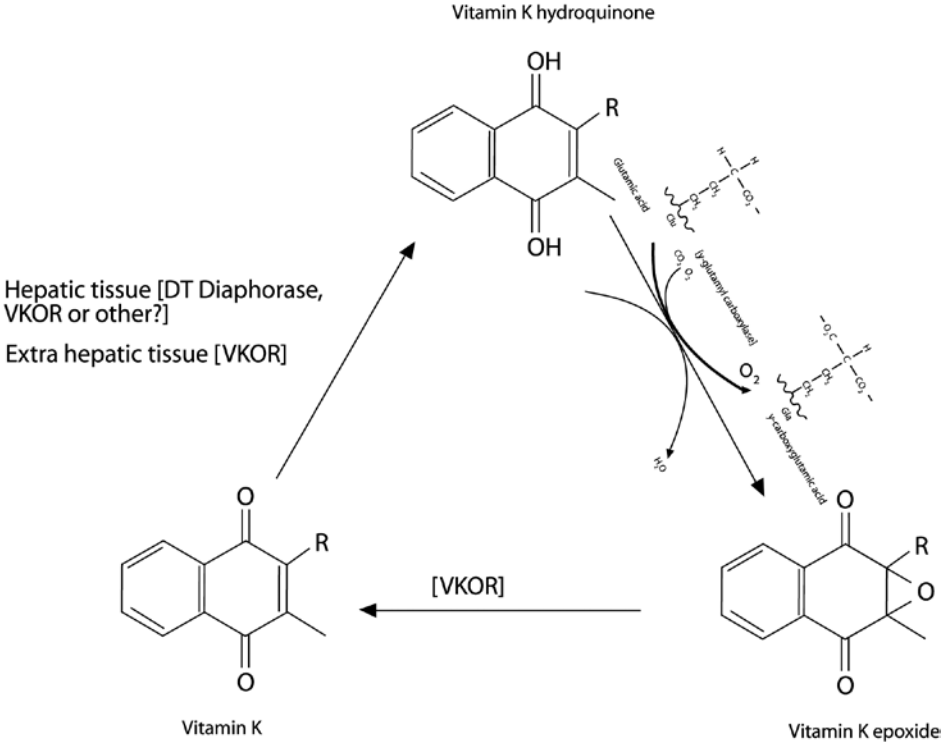


Figure 2 The vitamin K cycle



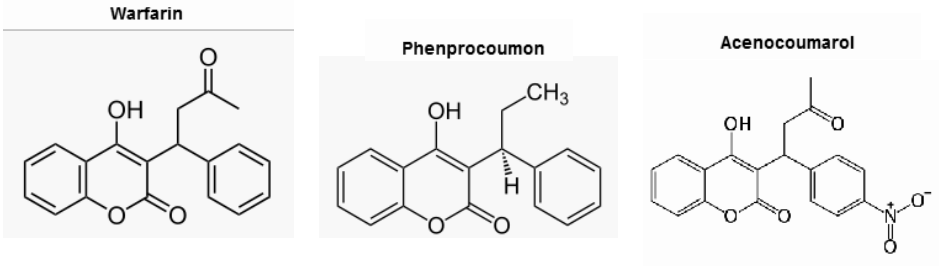
In hepatic tissue it is not clear if VKOR itself catalyzes the conversion of vitamin K to reduced vitamin K or if this is accomplished by a separate enzyme such as the DT diaphorase and possibly other enzymes. [16]

The carboxylation of the coagulation factors requires reduced vitamin K as a cofactor, which is oxidized to vitamin K epoxide in the process. Due to limited availability of vitamin K in tissues, the epoxide must be rapidly reduced again to vitamin K in what is known as the vitamin K cycle (figure 2).[6, 16] The enzyme for this reduction is called 'vitamin K epoxide reductase complex' (VKOR). Coumarins block this enzyme and prevent the regeneration of reduced vitamin K. This prevents the activation of coagulation factors II, VII, IX and X and thus stops the coagulation cascade. As this mechanism only effects the new development of these coagulation factors and does not have any effect on already carboxylated ones, the effect of coumarins is delayed until 72 to 96 hours after starting treatment. Therefore, the initiation of anticoagulant therapy is normally accompanied by heparins.

Coumarin chemistry and pharmacokinetics

The most commonly used coumarins are acenocoumarol, phenprocoumon and warfarin. In North America, the UK and in Scandinavia, warfarin is most frequently used, whereas acenocoumarol and phenprocoumon are the most commonly used coumarins in the other European countries. In the Netherlands, acenocoumarol is used most (1,100,000 prescriptions in 283,600 users in 2007) while phenprocoumon is used to a lesser extent (197,000 prescriptions in 72,000 users in 2007).[19] The choice is made by the referring physician and is often based on his or her experience with one of the two drugs.[17]

Figure 3 Chemical structure of coumarins



Chemically, the vitamin K antagonists belong to the group of 4-hydroxycoumarins that share a similar chemical structure (figure 3).[20] Each drug has a single, chiral centre that gives rise to two different enantiomeric forms, of which the S-form is approximately 2- to 5-fold more potent than its R-counterpart. In pharmacotherapy, the racemic 50:50 mixture of both enantiomers is administered orally. After rapid and almost complete absorption from the upper gastrointestinal tract, all coumarins are highly protein bound with low volumes of distribution.[20] They are metabolized in the liver by cytochrome P450 enzymes to mostly inactive metabolites, which are excreted in urine and feces.[21, 22] In principal, the more active (S-) enantiomers are mainly metabolized by cytochrome P450 CYP2C9.[20, 23] However, due to their differences in structure, there are differences between the pharmacokinetics of the three coumarin drugs. Phenprocoumon is metabolized for 60% and 40% is eliminated unchanged. Thus phenprocoumon is less dependent on biotransformation than warfarin and acenocoumarol. (S-)acenocoumarol undergoes extensive first pass metabolism and has a very low bioavailability in most patients. Therefore acenocoumarol effectiveness mainly depends on the less effective (R-)form. (R-) acenocoumarol is transformed for 50% by CYP2C9, for 30% by CYP1A2 and for 20% by CYP2C19.[20] Consequently the elimination half-lives of the coumarin anticoagulants vary widely and thereby the duration of effect. Table 1 gives an overview of the pharmacokinetics of the three coumarins.

Table 1: terminal elimination half-lives, main hydroxylation products, and main metabolizing CYP isoenzymes of the (S)- and (R)-enantiomers of warfarin, acenocoumarol, and phenprocoumon[a] [17]

Enantiomer	Elimination half-live (hours)	Hydroxylation product	Metabolizing CYP-isoenzymes	Ref
(S)-warfarin	24-33	4'-OH	2C8, 2C19	[55]
		6-OH	2C9	
		7-OH	2C9	
(R)-warfarin	35-58	4'-OH	2C8, 2C19	[55]
		6-OH	1A2, 2C19	
		7-OH	1A2, 2C8	
		8-OH	1A2, 2C19	
		10-OH	3A4	
(S)-acenocoumarol	1.8[b]	6-OH	2C9	[22]
		7-OH	2C9	
		8-OH	2C9	
(R)-acenocoumarol	6.6	6-OH	2C9	[22]
		7-OH	2C9, 1A2, 2C19	
		8-OH	2C9, 2C19	
(S)-phenprocoumon	110-130	4'-OH	2C8, 2C9, 3A4	[56]
		6-OH	2C9, 3A4	
		7-OH	2C9, 3A4	
(R)-phenprocoumon	110-125	4'-OH	3A4	[56]
		6-OH	2C9, 3A4	
		7-OH	2C9, 3A4	

Ref= reference, studies of the hydroxylation routes and contributing metabolizing enzymes in vitro; bold print = major metabolic pathway for the enantiomer or major metabolizing enzyme within a hydroxylation route

a Table is based on the comparative review of Ufer[56] and modified and supplemented by Schalekamp[17] according to the indicated references.

b Elimination half-life for the *CYP2C9*1/*1* genotype. In carriers of at least one **3 allele*, elimination half-life is increased to 9 hours in vivo.[57]

Monitoring of coumarin anticoagulant therapy

In order to achieve optimal treatment effects with coumarins, underanticoagulation with an exponentially increasing risk of thrombosis as well as overanticoagulation with its increased risk of bleeding have to be avoided.[24] This is difficult because of the narrow therapeutic range and large inter- and intrapersonal differences in dosage requirement over time. Conveniently, coumarin effectiveness can be directly monitored by the Prothrombin (PT) test, which is indicative for a reduction of the carboxylated clotting factors II, VII, and X.[25] Because the PT test requires thromboplastins which vary in their responsiveness to vitamin K dependent coagulation factors, PT values are not a suitable standard measure for anticoagulation.[17] For a standardized expression of the degree of anticoagulation, the International Normalized Ratio (INR) system has been adopted in which INR is assessed as follows:

$$INR=(\text{patient PT}/\text{ mean normal PT})^{ISI}$$

Table 2 Mean maintenance dosages of coumarins in Caucasians [20]

Coumarin	Maintenance dose (mg/day)
Warfarin	1.5 - 12
Acenocoumarol	1.0 – 9.0
Phenprocoumon	0.7 – 9.0

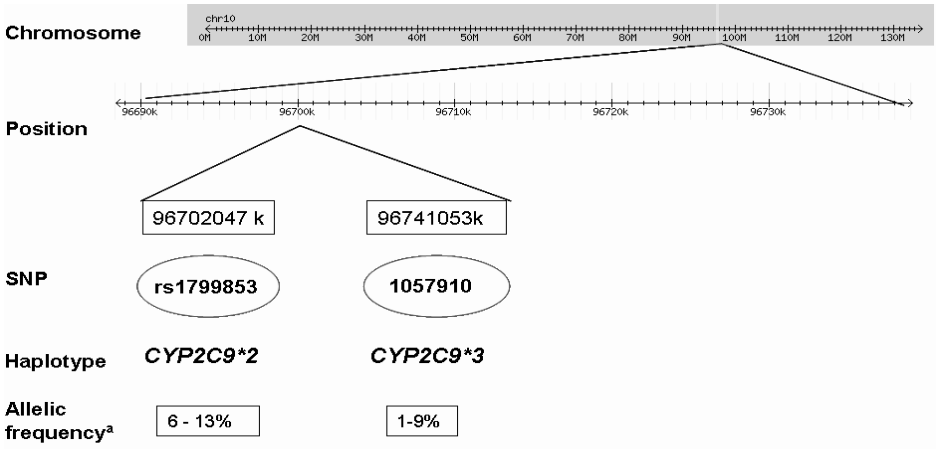
In this formula ISI is the International Sensitivity Index, a factor correcting for the responsiveness of the used thromboplastin and for the available instrument.[25] Use of the INR permits physicians to obtain the appropriate level of anticoagulation independent of laboratory reagents and to follow published recommendations for intensity of anticoagulation.[26] 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 [24, 27], depending on the indication for treatment. The necessary duration of treatment ranges from four weeks to lifelong.

In the Netherlands, there is a dense network of 61 regional anticoagulation clinics, monitoring about 300,000 patients.[17] There is evidence that management of anticoagulation by specialized clinics improved the quality of anticoagulation while saving costs by preventing major bleeding and thromboembolic events compared to usual medical care.[28] Although each anticoagulation clinic operates independently, many use one of several available computerized systems to assist with dosing schemes for coumarins.[26] These systems evaluate INR results and in about one-half of cases, they produce a dosage recommendation that can be followed by the physician[24]. 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. Table 2 shows the common range of maintenance dosage per coumarin. INR measurements and consequent adjustments of the dosing usually occur at intervals of 1 to 6 weeks, dependent on INR target range, the stability of the anticoagulant level and patient's co-medication.

Genetic factors affecting coumarin effectiveness

Coumarin maintenance dosage is influenced by age, gender, co-morbidity, vitamin K intake, and co-medication[25], but the highest amount of interpersonal dosage differences can be explained by genetic variation. In 1992, the cytochrome P450 isoform CYP2C9 was identified as the main metabolizing enzyme of the warfarin (S)- enantiomeric form.[29] The human gene coding for the CYP2C9 protein has been mapped on chromosome 10q24.2 and is greater than 55 kb in length.[30, 31] A total of 13 polymorphisms of the *CP2C9* gene have been identified of which two are used most to explain the enzyme activity (figure 4). The two most commonest *CYP2C9*2* and *CYP2C9*3*, occur at a frequency of 6-13% and 1-9% respec-

Figure 4 Gene structure of Cytochrome P450 isoform 2C9 (***CYP2C9***)

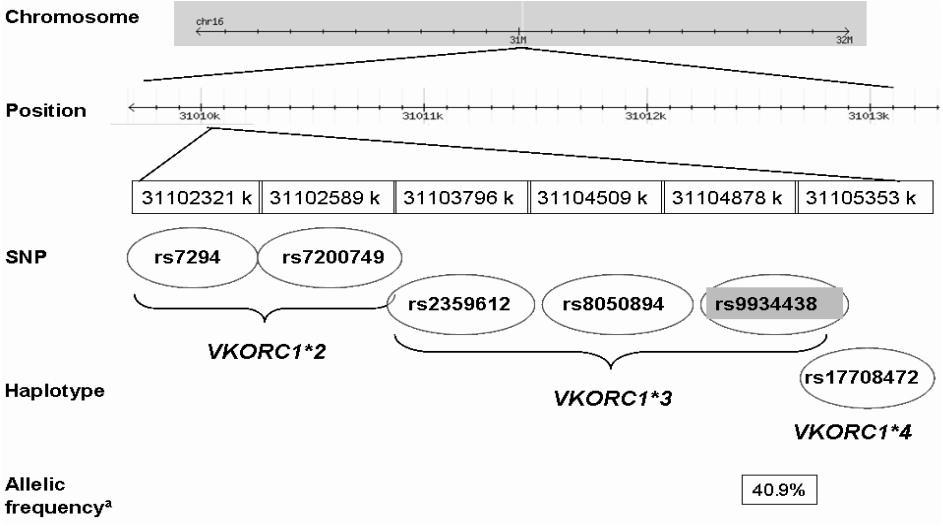


a Allelic frequency in Caucasians [32]

tively.[32] Variation in the *CYP2C9* gene explained 12% of warfarin dosage variation[33] and 6% of acenocoumarol dosage variation.[34] In relation to its lower dependency on CYP2C9 biotransformation, studies on the influence of CYP2C9 on phenprocoumon dosage gave inconsistent results.[35, 36]

In 2004, the gene *vitamin K epoxide reductase complex subunit 1* (*VKORC1*) was identified, which encodes for the crucial enzyme of the vitamin K cycle and which product is a direct target of coumarins.[9, 10] This gene is located on chromosome 16p11.2 and is approximately

Figure 5 Gene structure of Vitamin K epoxide reductase complex subunit 1 (*VKORC1*)



a Allelic frequency in Caucasians [47]

4 kb long (figure 5). Quite soon it was apparent that nearly all of the genetic variability of the *VKORC1* gene in Europeans is reflected by six single nucleotide polymorphisms (SNPs) which formed three main haplotypes and were in complete linkage disequilibrium.[37] Consequently one of these SNPs should be informative enough to explain differences in VKORC1 functionality.[38] In the Rotterdam Study, we genotyped rs9934438, the SNP that D'Andrea et al had identified as a marker for low dose warfarin requirement.[39] An important part of this thesis consists of studies regarding the contribution of this gene in addition to the already known influences of CYP2C9 in the Rotterdam Study.

The Rotterdam Study

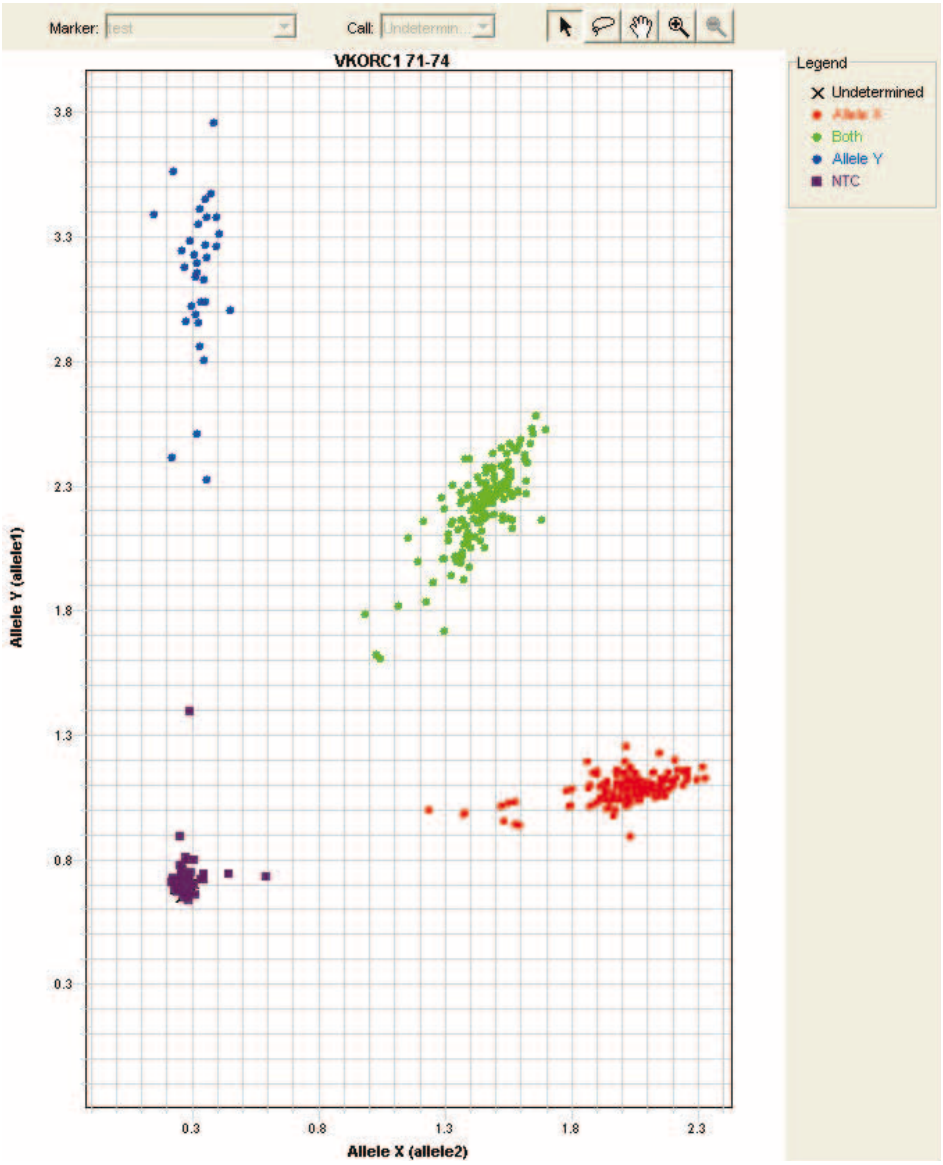
The Rotterdam Study (RS) is a large prospective population based cohort study of Caucasian subjects of 45 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands. The study was designed to investigate the incidence and determinants of neurological, cardiovascular, locomotor and ophthalmologic diseases.[40-42] Until now, three cohorts have been formed. The first (RS-I) cohort consisted of 7,983 subjects (response rate 78%) at baseline in 1990, the RS-II cohort consisted of 3,011 participants (response rate 67%) and was enrolled one decade later, and the recent RS-III cohort comprised 3,932 subjects (response rate 65%). All cohorts had baseline examinations with completed standardized questionnaires, sampling of blood and isolation of DNA and examinations such as ECG, echocardiography and laboratory assessments which were routinely repeated within the cohorts during up to five follow-up rounds.

All cohort members of the Rotterdam Study, treated with coumarins, are monitored by a regional anticoagulation clinic, the Star Medical Diagnostic Center. From this clinic, since 1984, all data on dosing, laboratory and clinical data, including data on bleeding complications are fully computerized. The patients own treating physician decides about the type of anticoagulant. Prothrombin times are monitored every 1-6 weeks, depending on the target level, stability of the INR and co-medication. Coumarin doses are adjusted on the basis of computerized dose calculations. More than 99% of RS I-III participants fill their drug prescriptions at seven regional pharmacies, which are fully computerised. Complete data on drug use from these pharmacies were available as of January^{1st}, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code[43], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs.

Candidate genes, genome-wide association studies and CHARGE

The first genetic studies used a candidate gene approach to demonstrate an association between genetic variation in a candidate gene and the risk of a certain phenotype. For instance, the polymorphisms in the *CYP2C9* gene and their effects on INR outcomes during coumarin treatment were largely discovered with a candidate gene approach following earlier clinical-

Figure 6 Taqman assay of *VKORC1 1173C>T* (rs9934438)



pharmacological kinetic studies.[44, 45] For a more detailed description of candidate gene analyses, the reader is referred to the study of Wadelius et al.[46] For *VKORC1* it soon became apparent that one SNP could reflect the activity of the enzyme.[37-39] One of them is *VKORC1* 1173C>T, rs9934438. To study the effects of genetic variation in the *VKORC1* gene, this SNP was determined in the RS on the basis of earlier literature.[39, 47] By means of a Taqman assay colour alleles could be distinguished into genotypes being homozygous for the most frequent allele, defined as the wild type genotype, being heterozygous and being homozygous for the variant allele (figure 6).

A limitation of candidate gene studies is that they only focus on associations with specific and *a priori* defined genes and often suffer from low statistical power, lack of replication and low precision.[48] To identify novel genetic loci, genome-wide association studies (GWAS) have to be performed, often without a prior hypothesis on biological mechanisms. They have proven to be successful in their ability to localize genetic effects in certain regions of the genome when they have adequate sample sizes and replication opportunities.[48]

Recently, it became technically and economically feasible to genotype in a single DNA sample more than 550,000 SNPs per person and to subsequently impute another 2 million SNPs per subject.[49] This has been done for all participants of the Rotterdam Study with DNA available which was the case in about 70% of the total cohort.

In the Single Nucletoide Polymorphism database (dbSNP) all genetic variation is documented, covering at the moment about 20 million SNPs.[50] The International HapMap project documents linkage relationships between SNPs by grouping those SNPs that are strongly correlated with each other into linkage blocks.[51] Currently in Caucasians about 1 million independent linkage blocks of correlating SNPs are known. With genome-wide association studies, associations between these linkage blocks and the phenotypes of interest can be estimated. Because of the multiplicity of testing so many markers, the number of false positive findings by chance increases.[51] Stringent thresholds have been considered to declare an association 'genome-wide significant'. The threshold agreed on for genome-wide significance is $5*10^{-8}$, which is the nominal p-value of the commonly used 0.05 divided by the number of independent linkage blocks.[52]

To confirm findings from a GWAS performed within one study population, they have to be replicated in another population. This is done by taking the 'top-hits' from the study population (for instance, all genetic markers above the significance threshold) and analyse the particular SNPs subsequently as candidate gene SNPs in the replication cohort. GWAS and replication studies often require 10 thousands subjects and for rare variants up to hundred thousands of subjects. Of course, this is more than a single cohort study can supply. Therefore international cooperation was needed with regular exchange of information on genotyping, validation of determinants and outcomes, and solving methodological questions. In 2008, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium was formed from three prospective cohort studies from the United States (the

Atherosclerosis Risk in Communities Study, the Cardiovascular Health Study, the Framingham Heart Study), and two cohorts from Europe (the Reykjavik Study and the Rotterdam Study). [48] This consortium facilitates GWAS meta-analyses and replication among multiple large and well-phenotyped cohort studies with DNA for all participants. Examples of milestones are the identification of genetic loci associated with a variety of conditions such as type 2 diabetes[53] and coronary heart disease[54]. At the moment, many meta-analyses on different disease outcomes are ongoing.

Aim and outline of this thesis

This thesis is a continuation of the research in an earlier thesis from our department in 2004 which demonstrated important variation in response to acenocoumarol and phenprocoumon in participants from the Rotterdam Study with different CYP2C9 genotypes.[26] In this thesis, the role of the *VKORC1* gene was studied in addition. With the discovery of the *VKORC1* gene in 2004 and SNPs that could explain the activity of the corresponding enzyme, it became possible to study the association between variant alleles responsible for reduced activity of this enzyme and outcomes such as anticoagulation and aortic calcification. Results from a study on the association between *VKORC1* variant alleles and aortic calcification are described in chapter 2.1. The additional influence of *VKORC1* variant alleles on acenocoumarol and phenprocoumon treatment with INR increase, bleeding and the need of dosage reduction as phenotypes are described in chapter 3 together with the results from GWAS in search for additional genetic factors with influence on coagulation therapy. Recent case reports on coumarin-drug interactions with PPIs and SSRIs and the lack of large cohort studies gave reason to study drug-drug interactions of acenocoumarol with proton pump inhibitors and selective serotonin reuptake inhibitors. The results are shown in chapter 4.1 and 4.2, and the role of the known genetic effect modifiers is part of it. Finally, in chapter 5 the main findings of these thesis are discussed and placed in a broader perspective.

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Chapter 2

Vitamin K Epoxide Reductase Complex Subunit 1 (*VKORC1*) polymorphism and aortic calcification: the Rotterdam Study

Abstract

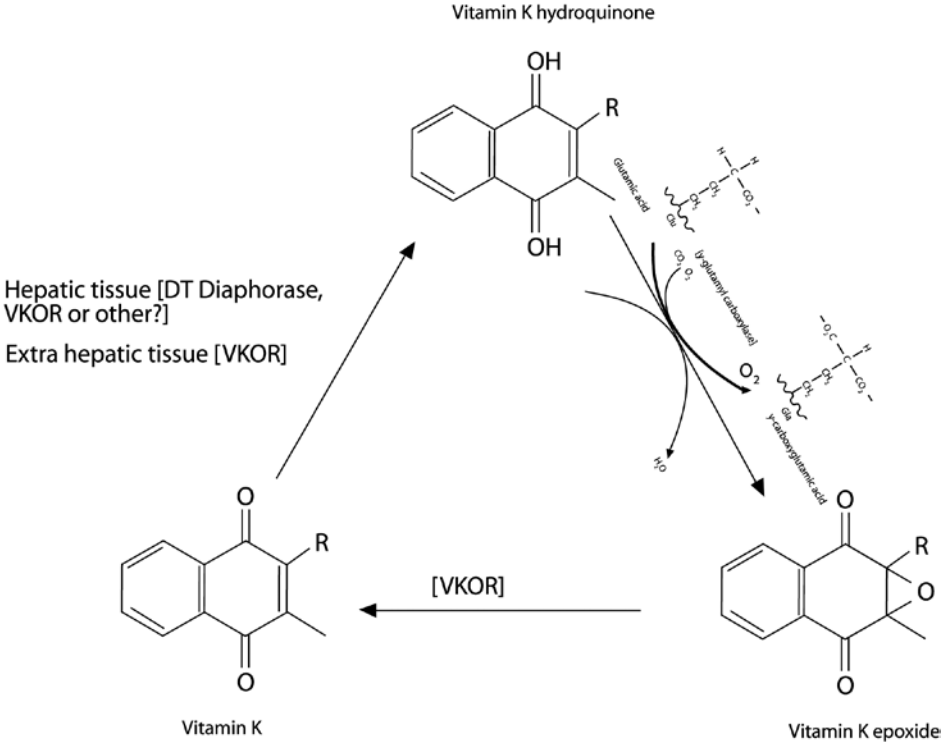
Besides effects on hemostasis, vitamin K-dependent proteins play a role in bone mineralization and arterial calcification. We investigated the association between the VKORC1 1173C>T polymorphism and calcification of the aortic far wall in a large population-based cohort. Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta. In all cohort members for whom DNA was available, the C1173T SNP of VKORC1 (rs9934438) was determined. With multivariable logistic regression analysis the association between this polymorphism and the risk of aortic calcification was calculated, adjusted for potential confounders. The T allele frequency of the VKORC1 1173C>T polymorphism was 38.8%. 1185 (37.2%) persons were homozygous CC, 1529 (48,0%) were heterozygous CT and 473 (14.8%) were homozygous TT. Persons with at least one T-allele had a statistically significant 19% (95% CI 2 to 40%) risk increase of calcification of the aortic far wall compared to CC homozygous persons, adjusted for age and gender. The T-allele of the VKORC1 1173C>T polymorphism was associated with a significantly higher risk of aortic calcification in Whites.

Introduction

Vitamin K epoxide reductase (VKOR) mediates recycling of vitamin K 2,3 epoxide to vitamin K hydroquinone, an essential cosubstrate for modification of multiple glutamic acid residues to γ-carboxyglutamate in vitamin K-dependent proteins such as the coagulation factors II, VII, IX, and X, protein C, S, and Z, Matrix Gla protein (MGP), and osteocalcin.[1] Recently, numerous single nucleotide polymorphisms (SNPs) were identified on chromosome 16 in the gene encoding the vitamin K epoxide reductase complex subunit 1 (VKORC1),[2, 3] of which several reflect 3 main natural haplotypes of VKORC1.[4-6] Five SNPs (rs 9934438, rs 9923231, rs8050894, rs 2359612, and rs 7294) were found to be in strong linkage disequilibrium (D'>0.9 and r^2 ≥0.9), indicating that any of these could reflect VKORC1 haplotypes.[5, 7] One of these SNPs, rs9934438 or VKORC1 1173C>T, is as informative about coumarin sensitivity as 5 VKORC1 haplotypes which predicted warfarin dose requirement and together accounted for 96% to 99% of the total haplotypes in European-American White populations.[5] The VKORC1 1173C>T SNP is likely to be one of the putative functional SNPs of the VKORC1 gene.[8]

The T-allele of this SNP modifies the effectiveness of coumarins, which reduce the activity of the VKORC1 enzyme.[4-6, 9-16] In carriers of the T-allele, additional inhibition by coumarins had a higher impact on hemostasis than in those with the 1173CC genotype. Beyond hemostatic effects, different studies suggest an influence of vitamin K-dependent proteins on bone mineralization and arterial calcification. The key function of MGP is to inhibit calcification in cartilage and arteries.[17-19] Hereto, MGP has to be activated by γ-carboxylation of its 5 glutamic acid residues, which is mediated by vitamin K hydroquinone. During carboxylation, the hydroquinone becomes oxidized to vitamin K epoxide (Figure). Vitamin K hydroquinone is derived from dietary vitamin K intake or by recycling of the epoxide. First, the epoxide is reduced to vitamin K, catalyzed by the Vitamin K epoxide reductase (VKOR). Second, vitamin K is further reduced to the hydroquinone. This second reduction step differs between tissues.[20] VKORC1 seems crucial for reduction of vitamin K in extra hepatic tissues, whereas in the liver also other enzymes such as DT diaphorase mediate further reduction of vitamin K into the hydrochinone.[21, 22] Inhibition of the VKORC1 with coumarins for coagulation factors could be antagonized by dietary vitamin K but not for MGP as extra hepatic protein. Price et al used the implication of this fundamental difference between tissues on the activation of vitamin K-dependent proteins by giving warfarin in combination with vitamin K to young rats. Thus mineralization of arteries could be promoted without inducing fatal bleeding before measurement of arterial calcification.[23] In human studies the recommended daily allowance for vitamin K was shown to be sufficient for maintaining functional hemostasis, whereas undercarboxylation of at least 1 nonhemostatic protein was observed.[18, 24] More recent studies showed that, despite their similar in vitro cofactor activity, the 2 forms of vitamin K differ concerning their ability to counteract effects of warfarin.[25, 26] High doses of vitamin K1 could counteract the effect of coumarins on coagulation factors in the liver but

Figure 1 The vitamin K cycle



not in extrahepatic tissue. In extrahepatic tissue only vitamin K2 was able to inhibit warfarin induced arterial calcification.[25]This implicates different effects of VKORC1 activity and of vitamin K1 and K2 intake on coagulation factors as hepatic proteins and on extrahepatic proteins such as MGP.

A diminished functionality of the VKORC1 enzyme is therefore not likely to influence coagulation factors and hemostasis in persons with normal vitamin K1 intake and not using coumarins. A lifelong decreased activity of the VKORC1 enzyme, however, might impair MGP activity and by this increase the risk of vascular calcification. This could be further worsened by reduced intake of vitamin K2. The association between impaired carboxylation of MGP and intimal and medial vascular calcification in humans has been described before.[27] Calcification of the aortic far wall has shown to be a good indicator of vascular calcification.[28] Therefore, we investigated whether carriers of the VKORC1 1173C>T allele had an increased risk of calcification of the aortic far wall in a large population-based cohort of Whites.

Methods

Setting

Data were obtained from the Rotterdam Study, a prospective population-based cohort study, designed to study neurological, cardiovascular, locomotor, and ophthalmologic diseases. The rationale and design of this study have been described elsewhere.[29, 30] Participants were visited at home for a standardized questionnaire and were subsequently examined at the research center. At baseline, information was obtained on several characteristics, including age, gender, smoking, blood pressure, diabetes mellitus, body mass index (BMI), medication use, measures of atherosclerosis such as vascular calcification, and a verified history of myocardial infarction and heart failure. During the first examination of the participants from 1990 to 1993, blood was taken and DNA was isolated, information on weight, height, morbidity, and blood variables was collected, and calcified deposits in the abdominal aorta were assessed by radiography.

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1 to 2 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS). We chose the 1173C>T SNP at intron 1, dbSNP: rs9934438. Genotyping was performed using a Taqman allelic discrimination assay as previously described.[31] To confirm the accuracy of genotyping results, 315 (5%) randomly selected samples were regenotyped with the same method. No inconsistencies were observed.

Outcome

In the present study, we used the measurements of calcified deposits in the aortic far wall taken at the baseline visits between 1990 and 1993. Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta as described elsewhere. The interobserver agreement for absence versus presence of atherosclerotic plaques was 0.88, and the κ statistic was 0.74.[32] We defined moderate and severe extent of calcification with an area of the posterior aortic wall involved >2.5 cm as the outcome of interest and used persons with absent aortic calcification as the reference group.

Radiography of the aortic far wall only measured calcified plaques and could not detect plaques without calcification. To check whether the VKORC1 1173C>T allele was associated with calcification independently from existing plaques, we performed further analyses with the data available on carotid plaques. With the ultrasonography used for the carotid measurements, it was possible to detect plaques composed of calcified as well as of noncalcified components. In the Rotterdam Study from 1990 until October 1991, with ultrasonography calcified and noncalcified plaques were assessed separately in the carotid artery as described elsewhere.[33] Assessment of calcified plaques was available from 3 locations in the carotid artery.

Cofactors

We adjusted for cardiovascular risk factors for atherosclerosis such as age, gender, present smoking, hypertension, hypercholesterolemia, and diabetes mellitus.[34] We further adjusted for nutritional vitamin K intake, separately for vitamin K1 and vitamin K2. The daily intake of these 2 forms of vitamin K was determined using a food frequency questionnaire.[35]

Statistical Analyses

Allele and genotype proportions were tested for deviations from Hardy-Weinberg equilibrium by a χ^2 test. For the 1173C>T SNP 3 different genotypes were present: CC, CT, and TT. With multivariable logistic regression analysis, adjusted for age and gender, we studied the association between the T allele and aortic calcification with an allele-dose-effect model (number of T-alleles with no T-alleles as a reference), genotype-effect model (genotype CT and TT separately with CC as a reference), a recessive model (TT versus CT plus CC), and with a dominant model (TT plus CT versus CC). We compared the risk of calcification of the aortic far wall to no detectable form of calcification as reference group. For each analysis an odds ratio (OR) and a 95% confidence interval (CI) was computed. Analyses were repeated for severe aortic calcification (area of plaque involved ≥5 cm) compared to no detectable form of calcification in the aortic far wall.

In multivariable logistic regression analyses we studied the association between the T-allele and calcification, adjusting for gender and age. Subsequently, we tested each risk factor for aortic calcification whether it changed the association between the T-allele and the outcome by more than 10%. Missing values for total serum cholesterol, dietary vitamin K1 and vitamin K2 intake, BMI, and systolic blood pressure were imputed with a predictive model that included age, gender, presence of the T-allele of the VKORC1 1173C>T polymorphism, and the presence of aortic calcification. We adjusted for missing values in present smoking, diabetes, and carotid plaques by computing 3 categories for each factor: measured present, measured absent, and measurement missing. We also checked for multiplicative interactions between the T-allele and each potential cofactor with interaction terms.

With multivariable logistic regression we analyzed the association of the T-allele with the presence of 4 to 6 calcified plaques in the carotid artery compared to no calcification detectable, adjusted for age and gender. To evaluate the independent risk estimate of calcification beside existing atherosclerotic deposits, we further adjusted for the total number of plaques (calcified or noncalcified). All statistical analyses were performed with SPSS software (version 11.0; SPSS).

Results

For 6547 of the 7983 persons in the Rotterdam Study a blood sample was available for genotyping of VKORC1 1173C>T. For 153 persons (2%) genotyping failed, leaving 6394 persons

with a genotype assessed. During the study period, 233 of the 6394 persons had used coumarins before measurement of aortic calcification and were excluded. Within the remaining 6161 patients, calcification of the aortic far wall was measured in 5123 persons during the first visit to the study center between 1990 and 1993. Measurements could not be evaluated for 123 persons, leaving 5000 persons. For 1667 persons no aortic calcification could be detected. In 1813 out of 3333 persons with plaques, the area of the aorta involved in the detected plaques was smaller than 2.5 cm whereas in the remaining 1520 persons the area of plaques involved a length of at least 2.5 cm. In the analyses we categorized persons without any detectable aortic calcification as calcification absent and those with plaques above 2.5 cm as calcification present. In total, our study population consisted of 3187 persons.

Characteristics of the study population are given in Table 1. Genotype proportions were similar to those in the whole population genotyped, and the population was in Hardy-Weinberg equilibrium (P=0.58).

Within persons with aortic calcification the numbers of the VKORC1 CT genotype were higher than in persons without detectable calcification (Table 2). In the study population, the

Table 1 Characteristics of the study population

Variable	Population (N= 3187)
VKORC1 1173 C>T	
Genotype (%)[$]	
CC	1185 (37.2)
CT	1529 (48.0)
TT	473 (14.8)
Allele frequency (%)	
C-1173	61.2
T-1173	38.8
Calcification of aortic far wall (%)	3187 (100)
Absent	1667 (52.3)
Present *	1520 (47.7)
Female gender (%)	1894 (59.4)
Age [years] (SD)	67.5 (8.0)
Body mass index [kg/m^2] (SD)	26.3 (3.6)
Systolic blood pressure [mm Hg] (SD)	138.8 (22.1)
Diastolic blood pressure [mm Hg] (SD)	73.9 (11.0)
Total cholesterol [mmol/l] (SD)	6.9 (1.2)
HDL-cholesterol [mmol/l] (SD)	1.4 (0.4)
Diabetes mellitus (%)	168 (5.3)
Smoking	
Current (%)	730 (23.1)
Past (%)	1329 (41.7)
History of myocardial infarction (%)	227 (7.1)
History of stroke (%)	93 (2.9)
Vitamin K1 intake (mcg) (SD)	250 (118)
Vitamin K2 intake (mcg) (SD)	28 (16)

$ Hardy-Weinberg Equilibrium $\chi^2 = 0.314$ (p= 0.58),

* involved area with calcified deposits of the posterior aortic wall with a length of at least 2.5 cm.

Table 2 Frequency of the VKORC1 1173C>T genotype within the groups of absent and present aortic calcification

Genotype (%)	Aortic calcification		Total
	Absent	Present*	
CC	654 (39.2)	531 (34.9)	1185 (37.2%)
CT	762 (45.7)	767 (50.5)	1529 (48%)
TT	251 (15.1)	222 (14.6)	473 (14.8%)
Total	1667 (100)	1520 (100)	3187 (100%)
Allele frequency (%)			
C-1173	62.1	60.2	
T-1173	37.9	39.8	

* Involved area with calcified deposits of the posterior aortic wall with a length of at least 2.5 cm

Table 3 Association between the VKORC1 1173C>T genotypes and the risk of mild, moderate and severe aortic calcification

	Number N=3187 (%)	Basic model[&] OR (95%CI)	Adjusted model$ OR (95%CI)
Allele-dose-effect model	3187 (100)	1.07 (0.95 – 1.19)	1.08 (0.96 – 1.21)
Number of T-alleles with 'no T-alleles' as a reference			
Genotype effect model			
CC	1185 (37.2)	1 (reference)	1 (reference)
CT	1529 (48.0)	**1.25 (1.05 – 1.47)**	**1.26 (1.06– 1.51)**
TT	473 (14.8)	1.04 (0.82 – 1.32)	1.05 (0.82 – 1.35)
Recessive model			
CC or CT	2714 (85.2)	1 (reference)	1 (reference)
TT	473 (14.8)	0.92 (0.74 – 1.14)	0.92 (0.74 – 1.16)
Dominant model			
CC	1185 (37.2)	1 (reference)	1 (reference)
CT or TT	2002 (62.8)	**1.19 (1.02 – 1.40)**	**1.21 (1.02 – 1.43)**

& adjusted for age and gender, $ adjusted for age, gender, total serum cholesterol, systolic blood pressure, current smoking, diabetes mellitus, BMI and vitamin K1 and vitamin K2 intake.

frequency of the T-1173 allele of the VKORC1 polymorphism was 38.8%. Persons with aortic calcification were found to have a higher T-allele frequency than persons with no detectable aortic calcification (39.8% versus 37.9%). From the models tested, only in the dominant model, persons with at least one T-allele had a statistically significant 1.19-increased risk (95% CI 1.02–1.40) for calcification of the aortic far wall compared to persons with the wild-type genotype of VKORC1 (Table 3). Systolic blood pressure, diabetes, present smoking, total serum cholesterol, BMI, and vitamin K1 and vitamin K2 intake did not change the point estimate by more than 10%, and for these variables no effect modification was observed for the association of the T-allele with aortic calcification. When adjusting for all these variables, the estimate for the T-allele slightly increased (1.21, 95% CI 1.02–1.43). Restricting the outcome to aortic calcification with an area of the posterior wall involved ≥5 cm instead of 2.5 cm, the risk in carriers of a T-allele was increased to 1.27 (95% CI 1.02–1.58).

Within the study population, there were 1084 persons with measurements of carotid calcification, 471 had no calcification detectable in the carotid artery and 75 persons had 4 to 6 calcified carotid plaques. In the carotid artery, the T-allele was not associated with a risk of 4 to 6 calcified plaques compared to no calcified plaques detectable (OR=0.68, 95% CI 0.44–1.07). When including the numbers of calcified plaques and noncalcified plaques together in analyses to evaluate the independent risk estimate of calcification given the total number of plaques, also no association was found for the T-allele with 4 to 6 calcified carotid plaques compared to no calcified plaques (OR=0.60, 95% CI 0.13–2.91).

Discussion

In our study, presence of the T-allele of the VKORC1 1173C>T was associated with a small but significantly increased risk of aortic calcification. To our knowledge this is the first study analyzing the association between the T-allele and aortic calcification. Persons with a T-allele have a lifelong reduced activity of VKORC1. Effects from this are expected in extrahepatic proteins such as MGP because γ-carboxylation here fully depends on VKORC.1 The estimated risk of aortic calcification for persons with the CT genotype (1 copy of the risk allele T) was higher than for those with the CC genotype (no risk allele). The risk of aortic calcification for homozygous persons with TT compared to CC individuals was not significantly increased, possibly because this group was much smaller with a less precise estimate and a wider 95% confidence interval. However, the point estimate for CT individuals was within the adjusted confidence interval of the TT individuals. This may be in line with a dominant effect of the T-allele, as already stated in another study.[7] Restricting the outcome to aortic calcification with an area ≥5 cm involved and its lower chance of misclassification, this was associated with a significant risk increase of 27% in the dominant model. So far as we know, there are few similar studies but with conflicting results.[7, 24]

Dietary vitamin K2 intake could have modified the association between the T-allele and aortic calcification. Previously, menopausal women in the Rotterdam Study with low dietary vitamin K intake had an increased risk of aortic calcification. In our study population, persons with aortic calcification had a significantly lower daily intake of vitamin K2 than persons without detectable aortic calcification (27.3 mcg/d versus 29.5 μg/d) However, there was no difference in dietary vitamin K intake in persons with or without a T-allele and further no interaction between a T-allele and dietary vitamin K2 intake was found. We also did not detect any confounding or multiplicative effect modification on this association by any of the known cardiovascular risk factors.

It is unlikely that our results can be explained by bias or confounding. Selection bias in this population-based setting is improbable, especially as the population was in Hardy-Weinberg equilibrium. In our study population we found a 38.9% allele frequency of the risk allele.

The frequency of the T-allele in Whites has been reported so far between 39.8 and 42%.[4, 6, 10, 11] The absence of significant deviation from the HWE is also an indicator of good SNP genotyping quality in our population. As our data were collected prospectively and independently from our research hypothesis, information bias is also unlikely. We adjusted for known cardiovascular risk factors as potential confounders, and these furthermore did not substantially change our risk estimates.

It is however possible that plaques already present in vessels might promote calcification and thus form an independent risk factor for aortic calcification. Vascular calcification can occur in the intima, always in the context of atherosclerosis, and in the media (Mőnckeberg's sclerosis) where it is independent of atherosclerosis and almost exclusively associated with vascular smooth muscle cells.[18, 27] Calcification in the media is very diffuse and was not detectable by radiography or ultrasonography. Radiography, which was used to measure the calcification in the aorta, could not detect noncalcified plaques. Sonography, however, could distinguish calcified plaques in the intima from plaques consisting of lipids or polysaccharides only. Such measurements were taken in our study population in the carotid artery during 1990 and October 1991. In contrast to the association between the T-allele and aortic calcification in our study population, the T-allele was not associated with an independent higher risk of 4 to 6 calcified plaques in the carotid artery. Repeating the analyses for carotid plaques in the whole population doubled the number of persons with available carotid measurements (884 with no carotid plaques detectable and 140 persons with 4 to 6 calcified carotid plaques) but did not change our results. A previous study within the Rotterdam Study had detected graded associations for coronary calcification with aortic calcification as well as with calcified carotid plaques.[28] In our study we found a high predictive value of calcified carotid plaques on aortic calcification (OR=3.05, 95% CI 2.61–3.56). However, cooccurrence of calcification in both vessels was quite low (Cohen's kappa=0.41), and this may explain why the association with aortic calcification was not seen in carotid arteries. Thus we could not verify our hypothesis that the VKORC1 1173C>T polymorphism increases the risk of calcification independently from the number of preexisting plaques. Yet this seems plausible, as in the animal experiment vascular calcification occurred in newborn rats which were unlikely to suffer from plaques already present.[17]

Our results may be important as vascular calcification is regarded as one of the major complications of cardiovascular disease.[36] It is now appreciated that the development of the atherosclerotic lesion ultimately causes plaque erosion and leads to rupture and to thrombus formation as one of the final events in atherosclerosis.[18, 37] On the other hand, in vitro studies for the impact of calcification on plaque stability showed protective rather than destabilizing influence on the atheromas.[38] This was in contrast to lipid pools that dramatically destabilized the plaques. Whether intimal calcification stabilizes atherosclerotic plaques or promotes its rupture is therefore still a matter of debate.[39]

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Chapter 3

Genetic influences on treatment with acenocoumarol and phenprocoumon

Chapter 3.1

Genotypes associated with reduced activity of *VKORC1* and *CYP2C9* and their modification of acenocoumarol anticoagulation during the initial treatment period

Abstract

The objective of this study was to investigate the influence of genotypes associated with reduced activity of vitamin K epoxide reductase complex subunit 1 (VKORC1) and cytochrome p450 2C9 (CYP2C9) on anticoagulation with acenocoumarol during the first 6 weeks of treatment. in 1,525 patients from the Rotterdam Study who were started on anticoagulation therapy with acenocoumarol, the presence of *VKORC1 1173C>T* and *CYP2C9*2* and **3* allele variants was determined. The first international normalized ratio (INR) after initial standard dose, risk of overanticoagulation, and mean dosage at the end of the initiation period were compared between genotypes. The initial standard dosage significantly increased the risk of severe overanticoagulation by 85% for each additional *VKORC1* T-allele present. at the end of the initiation period, each *VKORC1* T-allele present was shown to decrease the required acenocoumarol dosage by 5.1 mg/week, while each *CYP2C9* variant allele present reduced the required dosage by 1.8 mg/week. our conclusion was that an initial standard dosing regimen with acenocoumarol increases the risk of severe overanticoagulation in patients with variant alleles of the *VKORC1* and *CYP2C9* genes.

Introduction

Clinical management of anticoagulation with coumarins is difficult, as the target range must be achieved with the use of drugs that have a narrow therapeutic index but high intra- and interindividual variability in pharmacokinetic and pharmacodynamic response. Several recent studies have identified polymorphisms in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene that contribute most to the variability between patients in response to coumarins.[1-8] Other known genetic risk factors for this variability are polymorphisms of the cytochrome P450 isoform 2C9 (*CYP2C9*) gene.[9] Important non-genetic risk factors are age, heart failure, impairment of liver function, vitamin K intake, drug-drug interactions, smoking, fever and non-compliance.[10-14] Genetic variations in the *VKORC1* and *CYP2C9* genes have been shown to account for 30-50% of the variability in dosing of warfarin. In view of this, the Food and Drug Administration, in 2007, added to the product information for warfarin a note that genetic data might be relevant to prescribing decisions.[15] In 2008, an expert group concluded that there was insufficient evidence to recommend routine *CYP2C9* and *VKORC1* testing in warfarin-naïve patients and that prospective clinical trials were needed to provide evidence of the benefits and risks in the setting of initial warfarin dosing.[16] A recent review reached the same conclusion.[17] In the meantime, warfarin-dosage algorithms were developed and validated, and they are accessible online.[12, 18, 19] Recently, a pharmacogenetic approach was developed to allow clinicians to initiate warfarin therapy and then to change to a genetically predicted dose after 2-4 days of therapy.[18-20] However, this approach does not prevent the risk of severe overanticoagulation consequent to an initial standard dosage regimen in patients who are highly sensitive to coumarins.[21]

Given that most studies were performed using warfarin, we focused our study on acenocoumarol, a coumarin anticoagulant which is widely used in the Netherlands[22] and is effective in preventing thrombotic events.[23, 24] To date, only a few studies have been performed relating to the association of polymorphisms in the *VKORC1* and the *CYP2C9* gene to anticoagulation response during initiation of acenocoumarol[4, 25] and during maintenance dosage of this drug.[26] Carriers of variant alleles of either of these genes all showed an increased risk for severe overanticoagulation with standard dosages of the drug and needed lower maintenance dosages. These studies included between 113 and 231 Caucasian subjects and showed inconsistent results with respect to the arithmetical relationships between the two genes regarding dosage and the risk on severe overanticoagulation. Therefore, the objective of our study was to investigate the influence of variant alleles of the *VKORC1* and *CYP2C9* genes, independently and in combination, on the international normalized ratio (INR), severe overanticoagulation, and bleeding events after an initial standard dosage regimen of acenocoumarol in a large population-based cohort. In addition, the effect on severe overanticoagulation and the incidence of bleedings during the first 6 weeks of treatment and dosage at the end of initiation were evaluated.

Methods

Setting

Data were obtained from the Rotterdam Study, a prospective population-based cohort study designed to study neurological, cardiovascular, locomotor, and ophthalmologic diseases in a population of people ≥55 years of age. The rationale and design of this study have been described elsewhere.[27, 28] Participants were visited at home for a standardized questionnaire and were subsequently examined at the research center. During the first examination of the participants from 1990 to 1993, blood was taken and DNA was isolated. Additional patient information gathering, physical testing, and laboratory tests were performed at the first visit to the study center. A regional anticoagulation clinic monitors all inhabitants of Ommoord for indications requiring anticoagulant therapy. The choice of anticoagulant is made by the physician. All patients start with a standard dosing scheme of acenocoumarol (6-4-2 mg) from days 1 to 3. Prothrombin times are monitored every 1–6 weeks by reference to the INR depending on the stability of the anticoagulant level. Doses are adjusted on the basis of the INR of the patient via computerized calculations of required doses. Since 1984, collection of all data on dosing and on laboratory and clinical findings, such as data on bleeding episodes, is fully computerized.

Cohort definition

The study cohort consisted of all patients in the Rotterdam Study who started with acenocoumarol in the study period between 30 October 1985 and 8 December 2006 in whom genotyping for *CYP2C9* as well as *VKORC1* was successful and for whom an INR assessment was available within 4 days after the initial standard dosage of their treatment.

Outcomes

During this initiation period of acenocoumarol treatment, we distinguished three end points according to time points within the initiation period: the first INR assessment after initial standard dosage, the titration period of acenocoumarol during the first 6 weeks, and the end of the initiation period of 6 weeks. For these time-related end points, we estimated INR, severe overanticoagulation, bleeding episodes, or dosage as outcomes. The first end point at the first assessment after the initial standard dosage of acenocoumarol comprised the first INR as well as an INR≥6.0 and bleeding events. The second end point, at any time during the first 6 weeks of treatment, was an INR ≥6.0, or bleeding episodes (with distinction being made between minor and major episodes). Minor bleeds were skin bleeds and nosebleeds lasting for <10 min. Major bleeds were gastrointestinal, cerebrovascular, and urinary tract bleeds, and those within the eye or the female genital tract. The third end point, at the end of the initiation period of 6 weeks, was the mean dosage.

Genotyping

Two common SNPs in the CYP2C9 system are associated with impaired metabolism of coumarins.29 Genotyping for the *CYP2C9**2 and *CYP2C9**3 allele variants was performed using PCR followed by restriction enzyme digestion analysis, as previously described.[9, 29] All *CYP2C9**2 and *CYP2C9**3 heterozygote and homozygote variants detected were reanalyzed. Patients with neither *CYP2C9**2 nor *CYP2C9**3 alleles were regarded as having the wild-type genotype (*CYP2C9**1/*1). Allelic variants of *CYP2C9, CYP2C9**2 ($Arg^{144}Cys$), and *CYP2C9**3 ($Ile^{359}Leu$) code for enzymes with ~12 and 5% of the enzymatic activity of the wild-type genotype *CYP2C9**1/*1 (Arg^{144}/Ile^{359}), respectively. For genotyping of *VKORC1* variant alleles, we chose the 1173C>T SNP in intron 1, dbSNP: rs9934438. This SNP is likely to be one of the putative functional SNPs of the *VKORC1* gene and is recommended for genotyping.[30, 31] For carrying out the genotyping, genomic DNA was extracted from samples of peripheral venous blood in accordance with standard procedures.49 1–2 ng genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA). Genotyping was performed using a Taqman allelic discrimination assay, as previously described.[32] In order to confirm the accuracy of genotyping results, 315 (5%) randomly selected samples were re-genotyped using the same method. No inconsistencies were observed. Patients with the *VKORC1* CC genotype were regarded as having the wild-type genotype.

Statistical analysis

Allele proportions were tested for deviations from Hardy-Weinberg equilibrium, using a χ^2-test. The first INR after an initial standard dosage and the weekly dosage in those on acenocoumarol at the end of the initiation period were compared in *VKORC1* and in *CYP2C9* genotypes, using linear regression analysis adjusted for age and gender. For the weekly dosage at the end of initiation period, we further adjusted for target INR and usage of CYP2C9 co-medication. The following substrates and inhibitors of the CYP2C9 enzyme were considered as CYP2C9 co-medication: amiodarone, carbamazepine, chloramphenicol, cimetidine, diclofenac, fluconazole, fluvastatine, losartan, miconazole, phenylbutazone, phenytoin, sulfadiazine, sulfamethizole, sulfamethoxazole, sulfinpyrazone, tolbutamide, trimethoprim, and zafirlukast.[9]

All the genetic analyses described were performed with an allele-effect or genotype-effect model. Given the literature and confirmed by our findings (not shown), dominant/recessive models were not applicable. Subanalyses were performed for the *CYP2C9* alleles within the strata of the *VKORC1* genotype. Effect modification between variant alleles of the two genes was studied by adding multiplicative interaction terms to our model. Furthermore, the possibility of additive interaction was studied and expressed as excess relative risk reduction due to interaction.[33] The risk of experiencing an INR ≥6.0 after the initial standard dosage was analyzed using logistic regression analysis, adjusted for age and gender. These analyses were

performed using SPSS software, version 15.0 (SPSS, Chicago, IL). The risk on an INR ≥6.0 and the risk of experiencing bleeding episodes during the first 6 weeks of treatment were calculated by means of generalized estimating equation logistic regression analysis for repeated measurements, using STATA software, version 10.0 (StataCorp, College Station, TX) and also by means of Cox proportional hazard models in SPSS software, adjusted for age and gender.

Results

Among the 7,983 patients in the Rotterdam Study, genotyping showed polymorphisms in *VKORC1* in 6,624 individuals (80%) and of *CYP2C9* polymorphisms in 6,590 individuals (83%). A total of 6,355 patients (79.5%) had both *VKORC1* and *CYP2C9* genotyped. From the total of 2,531 individuals from the regional anticoagulation clinic who were using acenocoumarol during the study period, 2,310 were participants of the Rotterdam Study. Of these, 1,680 (73%) were genotyped for *VKORC1* as well as *CYP2C9* polymorphisms. We excluded 88 users who did not start with acenocoumarol but had been switched from phenprocoumon and 67 patients for whom no data were available on the first INR after the initial starting dosage. Consequently, 1,525 patients were included in our study population (**Table 1**). The mean age of these patients was ~76 years, and 58% were female. All patients were of Caucasian origin. The main indications for anticoagulation were short-term prophylactic treatment (very low target INR); short-term prophylactic treatment, deep venous thrombosis, pulmonary embolism, atrial fibrillation, and cerebral ischemia (low target INR); atrial fibrillation, myocardial infarction, vascular surgery, stroke, transient ischemic attacks, and peripheral artery disease (medium target INR); and prosthetic heart valves (high target INR). The frequency of occurrence of the *VKORC1* T-allele was 39.3%, of the *CYP2C9**2 allele, 13.4%, and of the *CYP2C9**3 allele, 5.7%. The cohort was in Hardy-Weinberg equilibrium for *VKORC1* 1173C>T SNP (P = 0.41) and also for the *CYP2C9**2 and *3 SNPs (P = 0.47 and P = 0.20, respectively).

The first end point was mean first INR, INR ≥6.0 and bleeding events after initial standard dosage. Measurement of the first INR after initial standard dosage was carried out on day 4 because this interval is considered to be sufficient to monitor the anticoagulation effects of acenocoumarol, whose effect starts within 18–24 h and reaches a maximum at 36–48 h after initiating treatment.[34] We considered an INR ≥6.0 as to be severe overanticoagulation, given that an INR above 6 units is associated with a greatly increased risk of bleeding.[35]

For the 1,525 patients who started on a standard dosing scheme with acenocoumarol, there was a statistically significant association between the mean first INR and the number of variant alleles for *VKORC1* (trend 0.33, 95% confidence interval (CI) 0.25–0.42, P value for trend <0.0001) and for *CYP2C9* (trend 0.13, 95% CI 0.06–0.19, P value for trend <0.0001) (**Table 2**). A direct comparison of heterozygous and homozygous *VKORC1* variant allele carriers with the wild-type CC genotype revealed a significant increase in the mean first INR in carriers of

Table 1 Characteristics of the study population

Number of patients	1,525
Age in years, average (SD)	75.8 (7.9)
Gender (%)	
Male	642 (42.1)
Female	883 (57.9)
Number of patients within a target INR level (%)*	
Very low INR: 2.0 – 2.5	58 (3.8)
Low INR: 2.5 – 3.5	881 (57.8)
Medium INR: 3.0 – 4.0	583 (38.2)
High INR: 3.5 – 4.5	3 (0.2)
Mean weekly dosage at the end of the initiation period (range) [mg]	15.9 (2 – 50)
Genotype *CYP2C9*, n # (%)	
CYP2C9*1/1	1,003 (65.8)
CYP2C9*1/2	321 (21.0)
CYP2C9*1/3	141 (9.2)
CYP2C9*2/2	30 (2.0)
CYP2C9*2/3	28 (1.8)
CYP2C9*3/3	2 (0.1)
Genotype *VKORC1*$ (%)	
VKORC1 CC	554 (36.3)
VKORC1 CT	743 (48.7)
VKORC1 TT	228 (15.0)

HWE: p-value for *VKORC1* T-allele = 0.410. P-value for *CYP2C9**2 = 0.472, p-value for *CYP2C9**3 = 0.197. In the study of Schalekamp et al the allelic frequency was 9.5% for the *CYP2C9**2 allele and 10.4% for the *CYP2C9**3 allele, for the *VKORC1* T-allele of the same SNP it was 40.9%.[4]
CYP2C9, cyotochrome P450 isoform 2C9; HWE, Hardy Weinberg Equilibrium; INR, international normalized ratio; VKORC1, vitamin K epoxide reductase complex subunit 1.
* In our study population target INR levels were mainly used with the following indications: very low target INR for short-term prophylactic treatment. Low target INR for short-term prophylactic treatment, deep venous thrombosis, pulmonary embolism, artrial fibrillation and cerebral ischemia. Medium target INR for artrial fibrillation, myocardial infarction, coronary bypass, vascular surgery, stroke, transient ischemic attacks, periphery artery disease. High INR target for prosthetic heart valves.
#Allelic frequency 80.9 %, for *CYP2C9**1, 13.4% for *CYP2C9**2 and 5.7 % for *CYP2C9**3.
$ Allelic frequency for the *VKORC1* C-allele 60.7% and for the T-allele 39.3%.

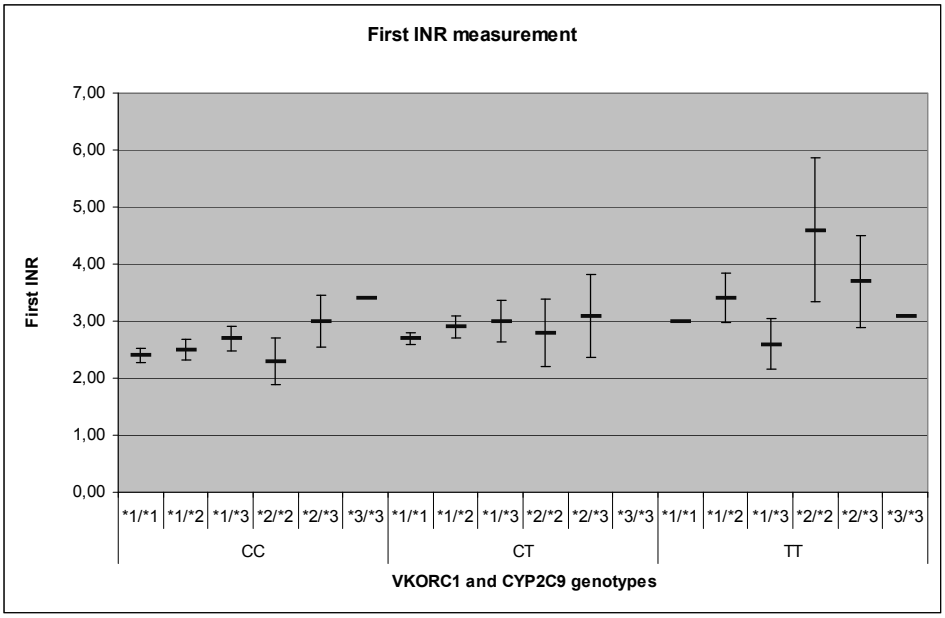
variant alleles, with 0.35 units of INR for the CT genotype (95% CI 0.22–0.48) and 0.66 units (95% CI 0.48–0.84) for the TT genotype, respectively. Within *CYP2C9* variant alleles, the mean first INR, as compared to the wild-type genotype, showed significant increases: 0.20 INR units (95% CI 0.05–0.35) for the *CYP2C9**1/*2 genotype, 0.49 INR units (95% CI 0.06–0.92) for **2/*2*, and 0.53 INR units (95% CI 0.08–0.97) for the *2/*3 genotype. No multiplicative interaction between variant alleles of was found for either of the genes. As shown in **Figure 1**, the risk of a high INR increased with the type and number of variant alleles. However, within the strata of *VKORC1* genotypes, the tendency of average INR to increase with increasing number of *CYP2C9* variant alleles was comparable, although averages were somewhat imprecise in the *VKORC1* TT stratum. Twenty-eight patients (2%) had a first INR ≥6.0 at the first assessment after an initial standard dosage (**Table 2**). The risk of an INR ≥6.0 after initial standard starting dosage rose significantly with the increase in the number of *VKORC1* T-alleles (OR

Table 2 After initial standard dosage with acenocoumarol: first INR and risk of severe overanticoagulation

Genotype	Number of patients within population	First mean INR (SE)	Difference with wild type genotype(95% CI) [a]	Number of patients with an INR≥6 at the first assessment (% of patients within genotype)	OR on INR≥6 at the first assessment (95% CI) [b]
CYP2C9	1,525		**Trend: 0.13 (0.06 – 0.19)**	28 (2.0)	Trend 1.26 (0.89 – 1.78)
*1/*1[c]	1,003	2.7 (0.04)	-	17 (1.7)	-
*1/*2	321	2.9 (0.07)	**0.20 (0.05 – 0.35)**	5 (1.6)	0.92 (0.34 – 2.53)
*1/*3	141	2.8 (0.10)	0.16 (-0.05 – 0.37)	3 (2.1)	1.25 (0.36 – 4.33)
*2/*2	30	3.2 (0.27)	**0.49 (0.06 – 0.92)**	2 (6.7)	4.08 (0.90 – 18.5)
*2/*3	28	3.2 (0.21)	**0.53 (0.08 – 0.97)**	1 (3.6)	2.21 (0.28 – 17.3)
*3/*3	2	3.3 (0.15)	0.52 (-1.13 – 2.16)	0 (0)	0
VKORC1d	1,525		**Trend 0.33 (0.25 – 0.42)**	28 (2.0)	**Trend 1.85 (1.08 – 3.16)**
CC	554	2.5 (0.04)	-	5 (0.9)	-
CT	743	2.8 (0.05)	**0.35 (0.22 – 0.48)**	16 (2.2)	2.43 (0.88 – 6.68)
TT	228	3.1 (0.09)	**0.66 (0.48 – 0.84)**	7 (3.1)	**3.54 (1.11 – 11.3)**

Statistically significant values are in bold printing
a linear regression model, adjusted for age and gender
b logistic regression model, adjusted for age and gender
c *CYP2C9* *1/*1 genotype is defined as wildtype genotype.
d *VKORC1* CC genotype is defined as wildtype genotype.

Figure 1 After an initial standard dosage with acenocoumarol: first INR



1.85, 95% CI 1.08–3.16). Patients with a *VKORC1* TT genotype had a 3.54-fold increased risk (95% CI 1.1–11.3) of an INR ≥6.0 after initial standard dosage as compared to patients with the *VKORC1* CC genotype. For *CYP2C9* there was a slight but nonsignificant trend toward an

INR ≥6.0 after initial dosage (OR 1.26, 95% CI 0.89–1.78) for each variant allele. Interactions of variant alleles of both genes could not be calculated, because among the subjects with a combination of a *VKORC1* wild-type genotype and a *CYP2C9* variant genotype there were no patients with an INR ≥6.0. We therefore repeated this analysis with the 67 patients with an INR ≥5.0 and compared the results with those of patients with an INR below 5.0. Again, no multiplicative or additive interaction was found for *VKORC1* and *CYP2C9* variant alleles (P = 0.123 for multiplicative interaction and the relative excess risk reduction due to interaction was 0.6 (95% CI −2.2 to 3.5)). Of the five bleeding events after initial standard treatment, one was minor and four were major bleeds. The minor bleed, a nosebleed for <10 min, was in a patient with the *VKORC1* CC genotype and the *CYP2C9* *1/*3 genotype. Of the major bleeds, one gastrointestinal bleed occurred in a patient who also had the *VKORC1* CC genotype and the *CYP2C9* *1/*3 genotype. The other two gastrointestinal bleeds and one vaginal bleed occurred in patients with the *VKORC1* CT genotype and the *CYP2C9* *1/*1 genotype.

The second end point was INR ≥6.0 and bleeding events during the first 6 weeks of treatment. The full first 6 weeks of treatment of the initiation period on acenocoumarol were completed by 1,521 patients. In total, there were 126 events of an INR ≥6.0 in 111 patients, representing an incidence rate of two events per 1,000 days of use. Repeated measurements analysis showed that there was a statistically significantly increase in the risk of occurrence of an INR ≥6.0 associated with an increase in the number of variant alleles of *VKORC1* (OR for trend 1.57, 95% CI 1.19–2.07) (**Table 3**) and of *CYP2C9* (OR for trend 1.28, 95% CI 1.04–1.56). For each of the genes, the homozygous variant genotypes were associated with a significantly increased risk

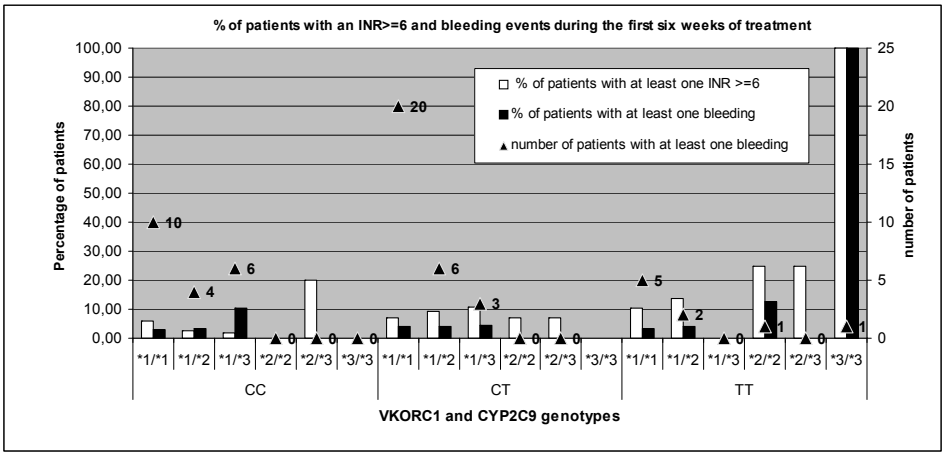
Table 3 During initiation period of six weeks: risk of bleeding and severe overanticoagulation

Genotype	Number of patients	Number INR measurements	Patients with at least one bleeding (%)	Combined genotype	OR bleedings [a] (95% CI)	OR on INR≥6 [b] (95% CI)	HR of the INR≥6 [c] (95% CI)
CYP2C9	1,521	8,939	58 (3.8)		Trend 0.98 (0.62 – 1.54))	**Trend 1.28 (1.04 – 1.56)**	Trend 1.36 (0.81 – 2.29)
*1/*1	1,002	5,864	35 (3.5)	*1/*1	-	-	-
*1/*2	320	1,896	12 (3.8)	*1/*2, *1/*3	0.93 (0.39 – 2.23)	1.17 (0.76 – 1.80)	0.92 (0.34 – 2.49)
*1/*3	139	830	9 (6.5)				
*2/*2	30	166		*2/*2, *2/*3, *3/*3		**2.73 (1.28 – 5.86)**	3.41 (0.79 – 14.8)
			1 (3.3)		0.65 (0.09 – 4.89)		
*2/*3	28	167	0 (0)				
*3/*3	2	16	1 (50)				
VKORC1	1,521	8,939			Trend 0.75 (0.41 – 1.38)	**Trend 1.57 (1.19 – 2.07)**	**Trend 1.86 (1.04 – 3.31)**
			58 (3.8)				
CC	552	3,296	20 (3.6)		-	-	-
CT	743	4,349	29 (3.9)		0.63 (0.26 – 1.51)	1.56 (0.98 – 2.48)	1.80 (0.63 – 5.11)
TT	226	1,294	9 (4.0)		0.69 (0.23 – 2.03)	**2.46 (1.42 – 4,.28)**	**3.44 (1.09 – 10.9)**

Statistically significant values are in bold printing

a GEE logistic regression model for repeated measurements, adjusted for age and gender; b logistic regression model, adjusted for age and gender; c COX proportional hazards model on INR≥6.0 and time to event, adjusted for age and gender

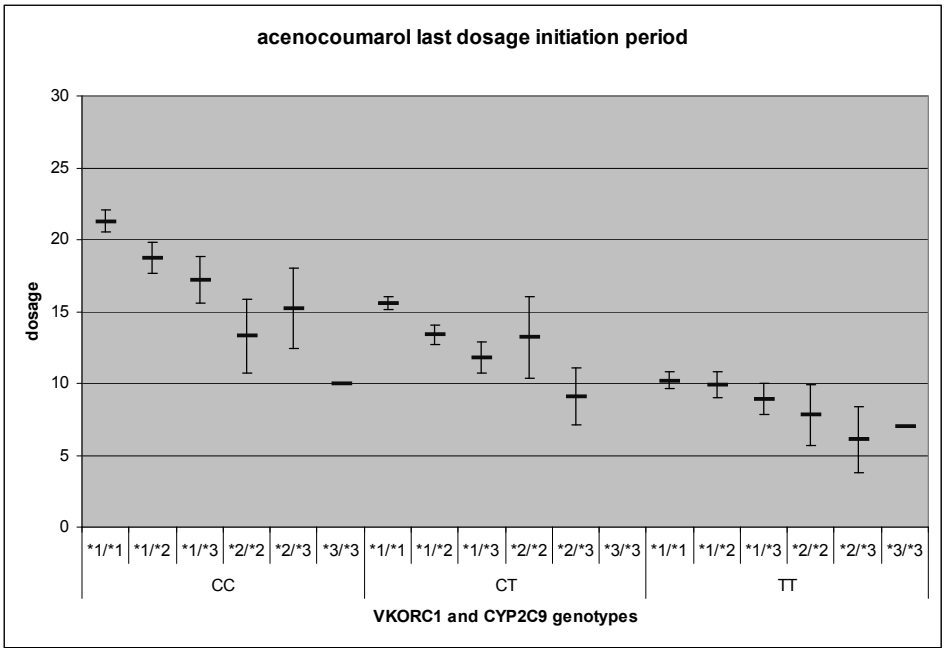
Figure 2 Percentage of patients with an INR≥6 and bleeding events during the first six weeks of treatment



of an INR ≥6.0. Also, in these analyses we did not find a significant multiplicative or additive interaction between the effects of the two genes. Cox proportional hazard models showed comparable hazard ratios, although with broader CIs, due to the fact that in these models only the first event was taken into account (**Table 3**). During the initiation period, 82 bleeding episodes were recorded in 58 patients, of which 40 were classified as major. The percentage of patients experiencing bleeding and severe overanticoagulation during the first 6 weeks of treatment increased with the number of variant alleles of both genes that were present (**Figure 2**). However, repeated measurements analysis did not detect any significantly increased risks for bleeding events in patients with variant alleles of either of the genes (**Table 3**).

The third end point was mean dosage at the end of the 6-week treatment period. We took the mean dosage at the end of the first 6 weeks of treatment. The steady state of a drug level is usually achieved within 5–7 half-lives of drug elimination. For acenocoumarol with a half-life of 2 days for the S-enantiomer, this period would be sufficient even if its elimination is prolonged in patients with *CYP2C9* variant alleles.[16, 30, 34] In the 1,521 patients who completed the 6-week initiation period on acenocoumarol, the dosage in mg/week had to be significantly titrated downward in association with an increasing number of *VKORC1* T-alleles (−5.1, 95% CI −5.5 to −4.7, *P* value for trend <0.0001) and of *CYP2C9* variant alleles (−1.8, 95% CI −2.1 to −1.5, *P* value for trend <0.0001) (**Table 4** and **Figure 3**). There was a significant multiplicative interaction between the effects of the genes on decrease of dosage ($P = 0.02$). All analyses were adjusted for age and gender. Repeating the analyses and adjusting additionally for target INR did not change the results. In our study population at the end of the initiation period, the *VKORC1* variant alleles explained 28% of the variation in the acenocoumarol dosage, whereas 5% of the dosage variation was due to *CYP2C9* genotype. A combined model, including age and gender, could explain 46% of the variation in acenocoumarol dosage at the end of the initiation period.

Figure 3 At the end of the initiation period: dosage of acenocoumarol [mg/week]



Discussion

The results of our study showed an unambiguous association between the first INR after an initial standard dosage of acenocoumarol and the presence of three genetic polymorphisms with a repeatedly demonstrated influence on coumarin and vitamin K metabolism.[1, 2, 4, 6, 7, 12, 25, 36-38] Initial INRs were consistently higher in patients with one or two variant alleles. After initial standard dosage, 2% of all the acenocoumarol-treated patients developed an INR that put them at a strongly increased risk of bleeding. The general assumption that patients can be safely treated by titration of dose on the basis of regular INR assessment may be too optimistic, given that the level of the first INR cannot be predicted without prior knowledge of the genotype status of an individual. It is also a likely explanation for the fact that, despite many efforts to bring down the risk, bleeding during the use of coumarin anticoagulants is still one of the leading causes of drug-related hospital admissions in most Western countries. [39, 40] In many European countries, for instance, France and the Netherlands, the coumarin anticoagulant acenocoumarol is used instead of warfarin. Of the 100,646 patients who were started on acenocoumarol in the Netherlands in 2007, 2% of bleeding episodes after initial standard treatment would correspond to more than 2,000 patients developing an INR ≥6.0 within 4 days after initial treatment. This might account for the substantial number of hospital admissions resulting from bleeding due to coumarins in the Netherlands.[41] Given that

the combination of age, gender, and polymorphisms of *CYP2C9* and *VKORC1* explains nearly 50% of acenocoumarol dosage variation, substantial improvements can be expected from a dosage algorithm that includes these variables. As long as the first dose of acenocoumarol is standard and only the second dose is titrated, the risk of overanticoagulation at the start of treatment will not decline. Only prior knowledge of the *VKORC1*- and *CYP2C9*-gene status of a patient may bring down this risk.

During the 6-week initiation period, each dosage following an INR measurement was titrated if not within the INR target range, resulting in a significant dosage decrease in those with *VKORC1* and *CYP2C9* variant alleles. Despite this, the risk of severe overanticoagulation remained higher in patients with variant alleles of either of the genes. When we adjusted for prior dosage in subanalyses of INR measurements, the effects of variant alleles on serious overanticoagulation were shown to be even stronger. This finding indicates that persons with variant alleles remain relatively unstable in comparison to those with the wild-type genotype, and that the step-by-step dosage titration is not sufficient to cope with this condition. Our results are in agreement with earlier studies that showed that variant alleles of *VKORC*1 and *CYP2C9* are associated with an increased risk of severe overanticoagulation in users of acenocoumarol and warfarin, respectively. These events occurred mainly in the early stages of treatment [4, 5, 7, 25] but persisted even after stabilization.[26] Furthermore, these studies showed that a prolonged time is required to achieve stability in patients with variant alleles. [42] We did not find an increased risk of bleeding episodes in patients with variant alleles of either of the genes. Although this finding is in agreement with the results of earlier studies of the effects of *VKORC1* and *CYP2C9* variant alleles during initiation with acenocoumarol, it may also be explained by a lack of power in the study design, given that the bleeding episodes are less frequent than the occurrence of an INR ≥6.0.[3, 43]

The modifications brought about by *CYP2C9*- and *VKORC1*-gene status on the effects of coumarins are regarded as being independent of each other rather than synergistic. [25, 30] However, no strict arithmetical relationship was found. Another study found a multiplicative interaction between variant alleles of the two genes with respect to the risk of severe overanticoagulation.[4] Our results did not confirm these findings. Instead, we found an independent additive effect of both genes on INR and severe overanticoagulation. However, we did find a multiplicative interaction between genotypes of the two genes for the mean dosage at the end of the initiation period. This might be because, in patients with the *VKORC1* CC genotype, variant alleles of *CYP2C9* had a higher impact on the mean dosage than in individuals with the variant *VKORC1* genotype. Thus, different studies show inconsistent arithmetic relationships between the two genes for dosage and severe overanticoagulation. Etiologically, however, it is less likely that the effects of variant alleles of *VKORC1* differ in the presence of variant alleles of *CYP2C9*, or vice versa, because the mechanisms of operation are very different. Linkage disequilibrium is unlikely because the *VKORC1* gene is located on chromosome 16p11.2 and the *CYP2C9* gene on chromosome

10q24, and their polymorphisms would therefore be expected to segregate independently in populations.[30]

The mean weekly dosage at the end of the initiation period might have been affected by the target INR. Moreover, interacting drugs could lead to changes being made in coumarin dosage. Although this is usually an effect modifier rather than a confounder, in the case of *CYP2C9*- metabolized drugs confounding might occur. However, adding these variables to our regression model did not change the results (Table 4). In subanalyses we further compared the number of dose adjustments needed during the first 6 weeks of the initiation period, and we did not detect any differences between the genotypes. We further studied whether patients had an increased risk of being below or above their target range. Here also, we did not detect any difference between the genotypes (data not shown). Despite the observational nature of our study, we think that the chance of bias and confounding is negligible. First, the Rotterdam Study is a prospective cohort study, and the regional anticoagulation clinic covered a complete area containing more than one million inhabitants in the Rotterdam region. Consequently, everyone who is treated as an outpatient with a coumarin anticoagulant will be registered as such, and selection bias is highly unlikely. This is confirmed by the fact that, in our study population, genotypes for both genes were in Hardy-Weinberg equilibrium. The allele frequencies of the variant alleles were comparable to results for the same SNPs in other Caucasian populations,[4] except that the frequency of occurrence of the *CYP2C9**3 allele was quite high in that study, whereas other studies showed lower allele frequencies for this SNP

Table 4 At the end of the initiation period: mean dosage of acenocoumarol

Genotype	Number of patients	Mean dosage at the end of initiation in mg/week	Difference with wild type genotype in mg/week (95% CI) [a]	Difference with wild type genotype in mg/week (95% CI)[b]	Difference with wild type genotype in mg/week (95% CI)[c]
CYP2C9[d]	1,521		**Trend –1.80 (-2.14-[-1.46])**	**Trend - 1.80 (-2.14 - [-1.46])**	**Trend -1.80 (-2.14 - [-1.40])**
*1/*1	1,002	16.9	-	-	-
*1/*2	320	14.8	**-2.27 (-3.03 - [-1.51])**	**-2.26 (-3.02 - [-1.50])**	**-2.27 (-3.02 - [-1.51])**
*1/*3	139	13.7	**-3.73 (-4.80 - [-2.66])**	**-3.72 (-4.79 - [-2.65])**	**-3.71 (-4.77 - [-2.64])**
*2/*2	30	11.8	**-5.06 (-7.25 - [-2.87])**	**-5.09 (-7.27 - [-2.90])**	**-5.12 (-7.31 - [-2.94])**
*2/*3	28	10.9	**-6.43 (-8.70 - [-4.17])**	**-6.44 (-8.71 - [-4.18])**	**-6.46 (-8.73 - [-4.20])**
*3/*3	2	8.5	**-9.43 (-17.8 - [-1.07])**	**-9.32 (-17.7 - [-0.95])**	**-9.44 (-17.8 - [-1.09])**
VKORC[e]	1,521		**Trend –5.09 (-5.47 - [-4.71])**	**Trend –5.09 (-5.47 - [-4.71])**	**Trend 5.08 (-5.46 - [-4.70])**
CC	552	20.1	-	-	-
CT	743	14.7	**-5.08 (-5.65 - [-4.51])**	**-5.08 (-5.65 - [-4.51])**	**- 5.09 (-5.66 - [-4.52])**
TT	226	9.9	**-10.2 (-11.0 - [-9.38])**	**-10.2 (-11.0 - [-9.38])**	**-10.2 (-11.0 - [-9.35])**

The first six weeks of treatment were taken as initiation period. Statistically significant values are in bold printing

[a] linear regression model, adjusted for age and gender. [b] linear regression model, adjusted for age, gender and target INR. [c] linear regression model, adjusted for age, gender, target INR and CYP2C9 co-medication. [d] *CYP2C9* *1/*1 genotype is defined as wildtype genotype. [e] *VKORC1* CC genotype is defined as wildtype genotype.

also.[44] Furthermore, another *CYP2C9* genotype, *1l, which is associated with the need for a lower warfarin dose, is not detected by the Taqman system.[45] This might cause misclassification of this genotype into the group of the *1 genotypes. However, irrespective of the direction of misclassification, such misclassification will be random and would therefore lead to conservative risk estimates rather than to falsely increased ones.

Because all data on exposure and diseases are prospectively gathered without knowledge of future research hypotheses, information bias is improbable. Furthermore, we adjusted for all known confounders, including the target INR range. Also, we expected few- if any -confounding in the association between genotypes and the outcomes in this study. As we had data only from the regional anticoagulation clinic, we might have missed information pertaining to patients with treatment initiation in a hospital. However, the exclusion of patients with hospital admissions prior to the start of coumarin therapy recorded by the local anticoagulation clinic did not change our results.

Personalized dosage of coumarins by genotyping prior to coumarin therapy might help to prevent serious adverse effects.[46] The feasibility of prospective application of gene-based coumarin dosing has been shown earlier, and adverse effects were fewer in patients with a personalized starting dosage than in those with a standardized starting dosage.[18, 46, 47] Additionally, it is important to investigate cost-effectiveness and the clinical utility of pharmacogenetic testing, keeping in mind that, although genotype-based dosage regimens reduce the risk of serious adverse effects, the delay in initiating therapy while awaiting genotyping could expose the patient to a higher risk of clotting.[30, 48] However, as initiation of coumarins is accompanied by therapy with low-molecular heparins, no serious harm should be expected in delaying treatment by a day. Initiation of a standard therapy and changing to a genetically adjusted dose algorithm after 2–4 days may be an alternative. The time and costs needed for genotyping are rapidly decreasing, while the accuracy, speed, and sophistication of diagnostic instruments are improving.[49, 50] If the level of the first INR can be modified by a genetically adjusted dose algorithm, this might contribute to safety in health care.

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Chapter 3.2

A genome-wide association study of acenocoumarol maintenance dosage

Abstract

Several genome-wide association studies have been performed on warfarin. For acenocoumarol, the most frequently used coumarin in many countries worldwide, pharmacodynamic influences are expected to be comparable. Pharmacokinetics however might differ. We aimed to confirm known or identify new genetic variants contributing to interindividual variation on stabilized acenocoumarol dosage by a GWAS. The index population consisted of 1451 Caucasian subjects from the Rotterdam study and results were replicated in 287 subjects from the Rotterdam study extended cohort. Both cohorts were genotyped on the Illumina 550K Human Map SNP array. From polymorphisms tested for association with acenocoumarol dosage, 35 single nucleotide polymorphisms (SNPs) on chromosome 16 and 18 SNPs on chromosome 10 reached genome-wide significance. The SNP with the lowest P-value was rs10871454 on chromosome 16 linked to SNPs within the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) ($P= 2.0 \times 10^{-123}$). The lowest P-value on chromosome 10 was obtained by rs4086116 within cytochrome P450 2C9 (*CYP2C9*) ($P=3.3 \times 10^{-24}$). After adjustment for these SNPs, the rs2108622 polymorphism within cytochrome P450 4F2 (*CYP4F2*) gene on chromosome 19 reached genome-wide significance ($P= 2.0 \times 10^{-8}$). On chromosome 10, we further identified genetic variation in the cytochrome P450 2C18 (*CYP2C18*) gene contributing to variance of acenocoumarol dosage. Thus we confirmed earlier findings that acenocoumarol dosage mainly depends on polymorphisms in the *VKORC1* and *CYP2C9* genes. Besides age, gender, body mass index and target INR, one polymorphism within each of the *VKORC1, CYP2C9, CYP4F2* and *CYP2C18* genes could explain 48.8% of acenocoumarol dosage variation.

Introduction

Clinical management of anticoagulation with coumarins is difficult, as a target range has to be achieved by drugs with a narrow therapeutic index and high intra- and interindividual variability in pharmacokinetic and pharmacodynamic response. In Caucasians, single nucleotide polymorphisms (SNPs) in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 2C9 (*CYP2C9*) genes are more strongly correlated with stabilized coumarin dosage than all other known patient-related factors.[1-11] Other candidate genes that are part of the vitamin K pathway, vitamin K dependent clotting factors or minor metabolic or transport pathways have been systematically screened and showed weaker associations with warfarin maintenance dosage.[12] Recently, a candidate gene study on warfarin dosing found that *CYP4F2* genetic variation explained an additional 2% of dosage variation.[13] This result was replicated.[14] As genetic variation in the *VKORC1* and *CYP2C9* genes in combination with age, gender and body mass index (BMI) accounted for 30–50% of the variability in dosing of warfarin, the Food and Drug Administration in 2007 extended the product labelling information of warfarin mentioning that genetic data might be relevant to prescribing decisions.[15] Most studies focused on warfarin, the worldwide mostly used coumarin drug. Acenocoumarol, which is the preferred coumarin in The Netherlands [16] and many other countries worldwide, has been studied for associations of polymorphisms in the *VKORC1* and the *CYP2C9* genes with outcomes in acenocoumarol treatment in four studies for initiation period [2, 17-19] and in three studies for maintenance dosage [1, 20, 21]. Both *VKORC1* and *CYP2C9* polymorphisms were not only independently correlated with acenocoumarol maintenance dose, but also with first international normalized ratio (INR) after initial standardized dosage, time to stabilized dose, time within the target therapeutic range and to bleeding events. It is expected that more reliable personalized coumarin dosing strategies could improve quality of care as well as enable safer treatment in more patients who would benefit from anticoagulation therapy.[22, 23] Therefore, it is important to determine whether there are common variants in genes which affect acenocoumarol dosage. Genome-wide association studies (GWAS) covering the majority of common variation in the human genome can be used in large samples to identify genetic variants that typically confer modest effect sizes for quantitative complex traits, defined in homogenous phenotypes, such as INR and coumarin dosage. The dose prediction models already developed for warfarin may not be applicable to acenocoumarol because of differences in pharmacokinetics between these coumarines.[24] A GWAS of acenocoumarol dosage either confirming already known or identifying new genetic influences may supply important information for the clinical trials on genotype predicted dosage being under way. Therefore, we conducted a GWAS in two independent cohorts to identify polymorphisms that could explain a large fraction of the variance in acenocoumarol maintenance dosage.

Materials and methods

Setting and inclusion criteria

This study was carried out within the baseline cohort of the Rotterdam study (RS-I), consisting of 7983 (response 78%). Subsequently, findings were replicated in the first extended cohort of the Rotterdam study (RS-II) with 3011 (response 67%) participants. The rationale and design of the Rotterdam study have been described elsewhere.[25, 26] In brief, the Rotterdam study is a prospective population-based cohort study, designed to study neurological, cardiovascular, locomotor and ophthalmological diseases in a population of people of 55 years and older. The RS-I cohort had baseline examinations during 1990–1993 with completion of standardized questionnaires, sampling of blood and isolation of DNA. The RS-II cohort consisted of a second independent cohort formed in 1999 with baseline examinations between 2000 and 2001.

A regional anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. This clinic covers the patients of the RS-I cohort as well as those from the RS-II cohort. The physician who treats the patient makes the choice of anticoagulant. Up to now, all patients start with a standard dosing scheme of acenocoumarol (6–4–2 mg) during day 1 up to day 3. Prothrombin times are monitored every 1–6 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 computerized dose calculations. Since 1984, all data on dosing, laboratory and clinical data, including data on bleeding complications are fully computerized.

From both cohorts, subjects were included in our study population if they were genotyped for GWAS, and if they had started with acenocoumarol in the study period between 30 October 1985 and 8 December 2006, and if they had an INR assessment within 4 days after initial standard dosage of their treatment. Patients who were switched to or from phenprocoumon during the first 6 weeks of acenocoumarol treatment were excluded.

Genotyping

RS-I cohort. Genomic DNA was extracted from whole blood samples using the salting out method [27]. Micro array genotyping was performed in the whole RS-I cohort with proper quality DNA samples (n =6449) using the Infinium II HumanHap 550K Genotyping BeadChipw version 3 (Illumina, San Diego, CA, USA). The Illumina 550K Bead Chip array was genotyped in all participants of the original Rotterdam study cohort with proper quality DNA samples (n =6449). Intensity files were analyzed using the Bead Studio Genotyping Module software v.3.1.14. A no-call threshold of 0.15 was applied to a custom-generated cluster file derived from the Illumina-provided cluster file (based on the cluster definitions applied to the HapMap CEPH cohort). In the custom-cluster file, 2308 SNPs with GenCall scores less than 0.90 were visually checked by two observes and manually reclustered or zeroed ac-

cordingly. Poorly performing samples with low call rate and 10th percentile GenCall score were excluded prior to calling genotypes. Any samples with a call rate below 97.5% (n = 209), excess autosomal heterozygosity >0.336 ~FDR <0.1% (n = 21), mismatch between called and phenotype gender (n = 36), or if there were outliers identified by the IBS clustering analysis (see below), clustering more than three standard deviations away from the population mean (n = 102) or if there were outliers identified by the IBS clustering analysis, clustering >3>97% (n = 129) were excluded from the analysis. In total, we disposed over 5974 analyzed samples having passed QC and inclusion criteria.

RS-II cohort. Genotyping was targeted in the whole RS-II cohort using the Infinium II HumanHap 550K Duo Genotyping BeadChipR version 3 (Illumina) as part of a large population-based project on genetics of complex traits and diseases, financed by the Dutch government through the Netherlands Scientific Organization—Large Investments (NWO Groot; 175.010.2005.011). Of the 3011 RS-II participants, 2613 (86.7%) gave consent for DNA analysis. Of these, 2548 (97.5%) were plated and included for the genome-wide genotyping. In the current study, we use the first set of RS-II samples made available on September 2008, which was genotyped successfully for 2020 samples of which 1895 remained for analyses after QC. We describe below the genotyping and QC processes for this first RS-II set. Genotyping procedures were followed according to Illumina manufacturer's protocols. All participants of the RS-II cohort with proper quality DNA samples (n ¼ 2611) were genotyped with the array. Intensity files were analyzed using the Beadstudio Genotyping Module software v.3.2.32. A no-call threshold of 0.15 was applied to a custom-generated cluster file derived from the Illumina-provided cluster file (based on the cluster definitions applied to the HapMap CEPH cohort). Poorly performing samples with very low call rate (90%) and 10th percentile GeneCall score were excluded prior to calling genotypes. Any samples with a call rate below 97.5% or pending to be processed (n = 528), excess of autosomal heterozygosity (F< 20.05; n = 5) mismatch between called and phenotypic gender (n = 14), or if there were genetic outliers identified by the IBS clustering analysis (clustering more than three standard deviations away from the RS-II population mean; n = 81) or with IBS probabilities more than 40% (n = 25) were excluded from the analysis; in total, 1895 samples were analyzed. Of the 1895 individuals with a mean age of 65.20 (SD 8.34) years, 1032 (54.45%) were women.

Genotyping of *CYP2C9**2 and *VKORC1* C1173C>T. In order to check LD with variant alleles of these two genes which were not included in the Illumina 550 array, we included the corresponding SNPs which had already been genotyped within both study cohorts and were used in earlier studies [18, 24]. Genotyping for *CYP2C9**2 allele variants was performed using the polymerase chain reaction followed by restriction enzyme digestion analysis (PCR-RFLP), as previously described [28]. For genotyping of the *VKORC1* 1173C>T SNP in intron 1, dbSNP: rs9934438, a TaqMan allelic discrimination assay was used as previously described [29].

Phenotype definition

The mean weekly acenocoumarol dosage at the end of the first 6 weeks of treatment was regarded as stable maintenance dosage. Steady state of a drug is usually achieved within 5–7 half-lives of drug elimination. The (S)-enantiomeric form of acenocoumarol has a 2–5-fold higher anticoagulant potency than the (R)-form. For acenocoumarol with a half-life of 2 h for the S-enantiomere, the initiation period of 6 weeks would be sufficient to reach steady state, even if its elimination was prolonged in patients with CYP2C9 variant alleles[30].

Statistical analysis

Basic model. The overall strategy involved linear regression analysis for the genotype–phenotype association of all SNPs within the Illumina 550 array in the index cohort and replication of the associations found in the second cohort. SNPs with a minor allele frequency, 0.01, a genotype call rate more than 95% or an exact HWE P-value less than 0.0001 were excluded from the analysis. We a priori declared results significant at $P < 5 \times 10^{-8}$, based on estimation of the multiple testing burden for GWAS of nearly all common variants in the human genome of European ancestry individuals for a target genome-wide $\alpha = 0.05$.[31] For replication of the specific SNPs associated with acenocoumarol dosage within the index cohort, in the replication cohort, a P-value less than 0.05 was considered significant. This was done first with a basic model for dosage at the end of 6 weeks initiation period as outcome, adjusted for the clinical factors age, gender and BMI and the patient's target INR. Target INR was included into our model in order to adjust for necessity of higher dosages due to intended therapeutic INR values. BMI was defined as (kg/m^2) and missing values were imputed with a regression model consisting of maintenance dosage as outcome with age, gender and target INR as variables. In order to adjust acenocoumarol maintenance dosage by these covariates, the unstandardized residuals from an additive linear regression model were used for genotype–phenotype associations in GWAS.

Extended model and meta-analysis. In order to detect SNPs within other genes independently associated with acenocoumarol dosage, we repeated GWAS in an extended model further adjusting for influences of *VKORC1* and *CYP2C9* as done previously in the GWAS of warfarin [32]. In the extended model, those SNPs were taken as independent co-variates that were found by the basic model to be the strongest associated SNPs from the *VKORC1* and from the *CYP2C9* locus. For the extended model, we performed a meta-analysis combining the results of both GWAS from RS-I and RS-II using the beta estimates with inverse variance weighting and genomic control to control for inflation.

Subanalyses. As acenocoumarol maintenance dosage is the result of dosage titration due to INR measurements, the first INR after standard dosage is an outcome more directly representing the reaction of a patient to the drug. Therefore, we repeated GWAS for the basic model in

subanalyses with the first INR after the initial standard dosage scheme of acenocoumarol at the fourth day of treatment as outcome. In order to adjust for influences of co-medication on the acenocoumarol maintenance dosage, we repeated the GWAS including also current use of substrates and inhibitors of the CYP2C9 enzyme besides the variables of the basic model in a second subanalysis. As CYP2C9 co-medication, the following drugs were considered: amiodarone, carbamazepine, chloramphenicol, cimetidine, diclofenac, fluconazole, fluvastatine, losartan, miconazole, phenylbutazone, phenytoin, sulphadiazine, sulphamethizole, sulphamethoxazole, sulphinpyrazone, tolbutamide, trimethoprim and zafirlukast.[28] In a third subanalysis, we repeated GWAS of acenocoumarol maintenance dosage adjusting for vitamin K intake at baseline besides the variables of the basic model.

Regression model on dosage for clinical impact. In a multivariate linear regression model, the additional contribution of newly found genetic factors was assessed by the adjusted r^2 (r^2adj). The r^2adj statistic measures the proportion of the total variability explained by the model with adjustment for the number of parameters in the model.

Software

For the genome-wide association analysis, we used PLINK v.1.04 (59). The meta-analysis was performed using METAL. http://www.sph.umich.edu/csg/abecasis/Metal/) Formultivariate linear regression analysis, we used SPSS software (version 15.0).

Supplementary material

Supplementary Material is available at HMG online.

Results

Index and replication cohorts

For the index population from the total of 7983 subjects in the RS-I cohort, 5974 (75%) subjects were successfully genotyped and passed quality control (QC) for GWAS. Of these 5974 subjects, 1451 (24%) started acenocoumarol therapy at a standard initial dose and had a first INR measurement at the fourth day available. Of the 3011 subjects in the RS-II cohort 1895 persons (63%) were successfully genotyped for GWAS and 287 subjects (15%) started with acenocoumarol during the study period and had an INR measurement on day 4 after standard initial dose. Owing to study design, subjects in the replication cohort were on average 7 years younger and the percentage of male subjects was higher than in the RS-I cohort (50% versus 43%, Table 1). For BMI in the RS-I cohort, values for 39 persons (2.8%) had to be imputed and for the RS-II cohort, one value (0.3%) was missing. On average, BMI was slightly higher in the replication cohort. In our study populations, acenocoumarol was

Table 1 Characteristics of the RS-I and of the RS-II cohort

	RS-I cohort	RS-II cohort
Number of patients	1451	287
Age in years, average (SD)	75.9 (7.9)	68.7 (9.5)
Gender (%)		
Male	620 (42.7)	144 (50.2)
Female	831 (57.3)	143 (49.8)
Body Mass Index (BMI), average (SD)	26.9 (3.8)	27.9 (3.9)
Number of patients within a target INR-level (%):		
Very low: 2.0 – 2.5	54 (3.7)	23 (8.0)
Low: 2.5 – 3.5	840 (57.9)	171 (59.6)
Medium: 3.0 – 4.0	555 (38.2)	90 (31.4)
High: 3.5 – 4.5	2 (0.1)	3 (1.0)

prescribed with a very low target range (2.0–2.5) for short-term prophylactic treatment, with a low target INR range (2.5–3.5) also in patients for short-term prophylactic treatment, for deep venous thrombosis, pulmonary embolism, atrial fibrillation and cerebral ischemia, with medium target INR range (3.0–4.0) in patients with atrial fibrillation, myocardial infarction, vascular surgery, stroke, transient ischemic attacks and periphery artery disease and with a high target INR range (3.5–4.5) for patients with prosthetic heart valves (Table 1).

Polymorphisms associated with acenocoumarol dosage

Basic model. In the RS-I cohort, only SNPs on chromosome 16 and chromosome 10 were in genome-wide significant association with acenocoumarol dosage at the end of initiation period, adjusted for age, gender, BMI and target INR (Table 2 and Fig. 1). The 15 SNPs with P-values lower than 10^{-28} were all located on chromosome 16. Best associated, with a P-value of 2.0×10^{-123}, was rs10871454, located in the Syntaxin 4 A–placental (*STX4A*) gene, flanking the *VKORC1* gene (Fig. 2). Each additional minor allele of this SNP showed a decrease of weekly acenocoumarol dosage with 5.2 mg/week. This SNP was in linkage disequilibrium (LD) (r^2=1.0) with rs889548 scoring second in significance, with the third SNP, rs1978487 (r^2 =0.93) and to a lesser extent with the fourth SNP rs749767, r^2 = 0.26. One SNP, rs7294,

Table 2 GWAS of acenocoumarol maintenance dosage, basic model

Chromosome	SNP	Gene	Base pair position	RS-I cohort				RS-II cohort P-value
				Subjects	Beta[a]	$R^{2\,b}$	P-value	
16	rs10871454	STX4A	30955580	1451	-5.2	0.3198	**$2.0*10^{-123}$**	**$1.4*10^{-24}$**
10	rs4086116	CYP2C9	96697192	1450	-2.9	0.06871	**$3.3*10^{-24}$**	**$1.6*10^{-6}$**

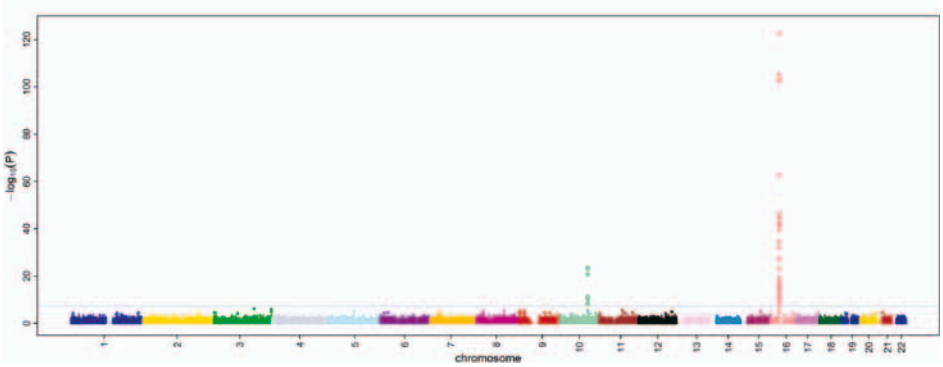
The SNPs with lowest p-values each within chromosome 16 and within chromosome 10 are reported. For all 53 genome-wide signficant SNPs from the basic model we supply an online supplement for complete overview of all genome wide significant SNPs from the basic model. Basic model: adjusted for age, gender, BMI and target INR, significant results are printed in bold.

Abbreviations: *STX4A* (Syntaxin 4A, placenta). *CYP2C9* (Cytochrome P450 2C9)

a dosage change of acenocoumarol in mg/week per additional variant allele

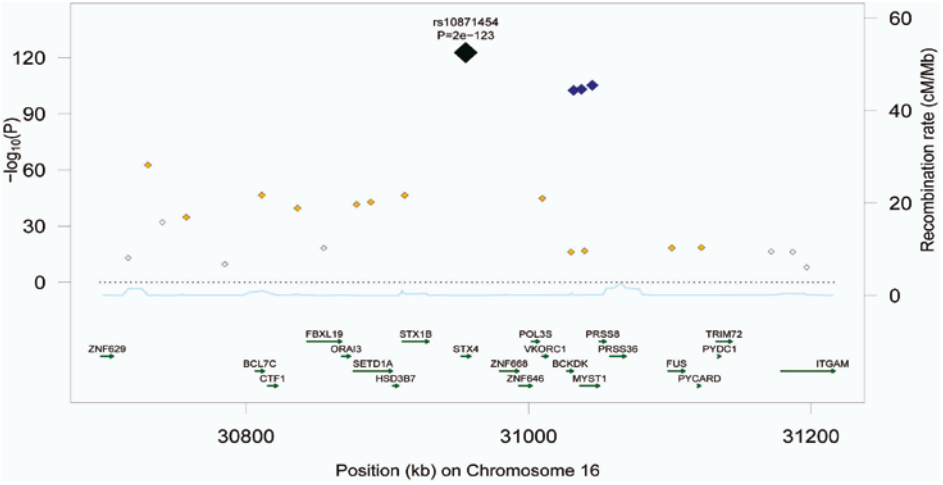
b Univariate r^2 between acenocoumarol maintenance dosage and the specific SNP.

Figure 1 Genome wide p-values for acenocoumarol dosage association in the RS-I cohort, basic model



All p-values are shown for univariate effects using an additive linear regression model, adjusting for age, gender, BMI and target INR. Chromosomes are numbered on the x-axis. Genome-wide significance was set at the p-value 5×10^{-8} (see line). Evidently only polymorphisms within chromosome 16 and chromosome 10 passed this threshold.

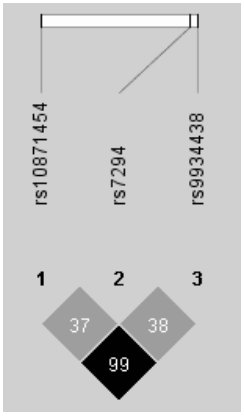
Figure 2 Position of the SNP on chromosome 16 with the lowest p-value on acenocoumarol dosage in the RS-I cohort, basic model



Observed p-values (-log p) are shown for univariate effects of polymorphisms within 500 kb of rs10871454 using an additive linear regression model in the RS-I cohort, adjusted for age, gender, BMI and target INR. Colour legend: black: the index SNP with the lowest P-value from our analysis; red: SNPs with linkage disequilibriumof r^2=1.0 to the index SNP; blue: 0.8<= r^2<=1.0; yellow: 0.5<=r^2<=0.8; orange: 0.2<=r^2<=0.5 and white: r^2<0.2.

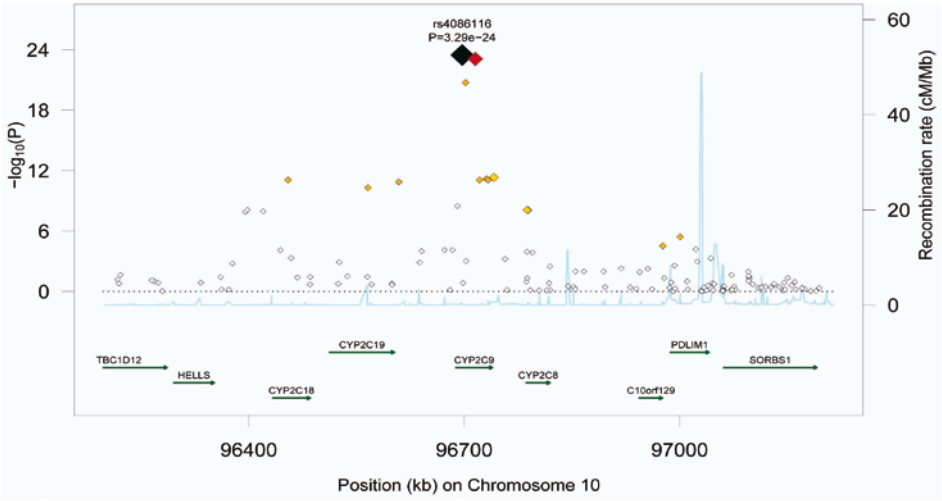
was located within the *VKORC1* gene and in some LD with rs10871454 (r^2 = 0.36, Fig. 3) and reached genome-wide significance at a P=1.4×10^{-45}. The SNP rs10871454 was in complete LD in our RS-I cohort with rs9934338 (*VKORC1 1173C>T*, r^2 = 0.99), one of the two SNPs that were able to predict the influence of VKORC1 on warfarin dosage [6, 33] and possibly one of the two causal putative SNPs within the *VKORC1* gene (5) (Fig. 3).

Figure 3 Linkage disequilibrium of the SNPs on chromosome 16 for VKORC1 within the RS-I cohort



The LD-plot with R^2 values is shown for **rs7294** (SNP within the *VKORC1* gene), **rs10871454** (SNP with the lowest p-value from GWA, basic model). **rs9934438** (SNP *VKORC1 1173C>T*, possibly one of the putative functional SNPs within *VKORC1*).

Figure 4 Position of the SNP on chromosome 10 with the lowest p-value on acenocoumarol dosage in the RS-I cohort, basic model



Observed p-values (-log p) are shown for univariate effects of polymorphisms within 500 kb of rs4086116 using an additive linear regression model in the RS-I cohort, adjusted for age, gender, BMI and target INR. Colour legend: black: the index SNP with the lowest P-value from our analysis; red: SNPs with linkage disequilibriumof r^2=1.0 to the index SNP; blue: 0.8<= r^2<=1.0; yellow: 0.5<=r^2<=0.8; orange: 0.2<=r^2<=0.5 and white: r^2<0.2.

The second locus reaching genome-wide significance was located on chromosome 10 (Table 2). The SNPs with the lowest P-values were rs4086116 (P= 3.3×10^{-24}, Fig. 4) and rs4917639 (P= 8.0×10^{-24}), both located within *CYP2C9* and in complete LD with each other

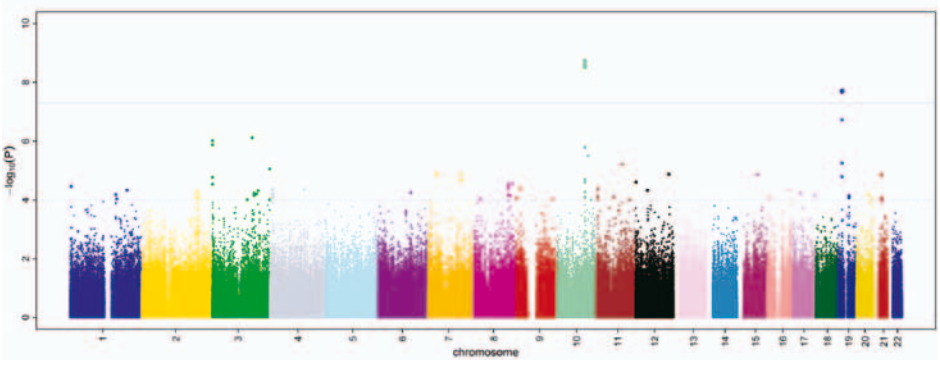
(r^2 =1.0). Our Illumina array included the *CYP2C9**3 SNP (rs1057910), reaching a genome-wide significant association with acenocoumarol dosage at a P =.6.44 × 10^{-12}. More SNPs within or flanking the *CYP2C18, CYP2C19* and *CYP2C8* genes reached genome-wide significance.

In our replication cohort RS-II, all SNPs on chromosome 16 were convincingly replicated (Table 2, Supplementary Material online). The two SNPs on chromosome 10 within CYP2C9 scoring lowest for genome-wide significance in the RS-I cohort were replicated in the RS-II cohort with P-values less than 10^{-6}, which is not genome-wide significant but yet far below the threshold of significance for replication (Table 2, Supplementary Material online). Further SNPs for *CYP2C18* and *CYP2C19* were replicated (P-values 10^{-6}), but not for *CYP2C8* (P =0.11).

Subanalysis. For GWAS on the first INR after initial standard dosage of acenocoumarol, P-values of the basic model within the RS-I cohort were substantially higher, with rs10871454 reaching a P < 10^{-15} instead of P < 10^{-123} for dosage at the end of initiation period as an outcome. Genome-wide significant values were only obtained for 11 SNPs from chromosome 16 and thus no other genes besides *VKORC1* were associated with INR after standard initial dosage of acenocoumarol. The other subanalyses on maintenance dosage adjusting for CYP2C9 co-medication or for vitamin K intake did not change our results.

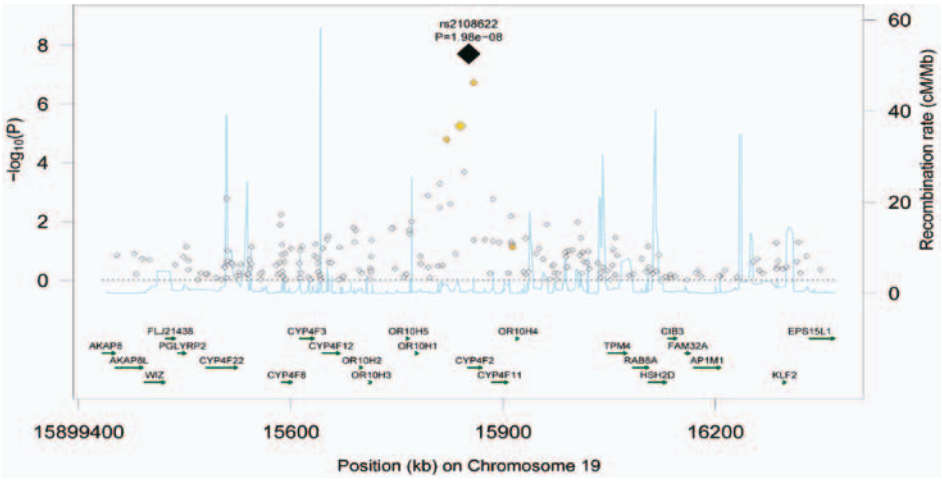
Extended model. In the extended model, we included the SNPs with the lowest P-values from the basic model for *VKORC1* (rs108714540) and *CYP2C9* (rs4086116) as independent covariates (Table 3 and Fig. 5). After adjustment for the influence of these two genes on variation of acenocoumarol dosage, only three SNPs on chromosome 10 (rs1998591, rs2104543, rs12772169), all flanking the *CYP2C18* gene, and one SNP in chromosome 19 within the

Figure 5 Genome wide p-values for acenocoumarol dosage association in the RS-I cohort, extended model



All p-values are shown for univariate effects using an additive linear regression model, adjusting for age, gender, BMI, target INR and rs10871454 and rs4086116 as independent covariates in the extended model. Chromosomes are numbered on the x-axis. Genome-wide significance was set at the p-value 5 × 10^{-8} (see line). Evidently only polymorphisms within chromosome 10 and chromosome 19 passed this threshold.

Figure 6 Position of the SNP on chromosome 19 with the lowest p-value on acenocoumarol dosage in the RS-I cohort, extended model



Observed p-values (-log p) are shown for univariate effects of polymorphisms within 500 kb of rs2108622 using an additive linear regression model in the RS-I cohort, adjusted for age, gender, BMI, target INR and rs10871454 and rs4086116 as independent covariates in the extended model. Colour legend: black: the index SNP with the lowest P-value from our analysis; red: SNPs with linkage disequilibriumof r^2=1.0 to the index SNP; blue: 0.8<= r^2<=1.0; yellow: 0.5<=r^2<=0.8; orange: 0.2<=r^2<=0.5 and white: r^2<0.2.

CYP4F2 (rs2108622) gene reached genome-wide significance (Fig. 6). These four SNPs were replicated in our second cohort (Table 3). The three SNPs flanking *CYP2C18* had also reached genome-wide significance in the basic model, whereas rs2108622 within *CYP4F2* only had reached a P =7.0 × 10^{-5} in the basic model.

The acenocoumarol dosage variance for the *CYP2C19* SNPs could partially, though not fully, be explained by the SNP rs4086116 within *CYP2C9*: the P-value for rs3862009, the *CYP2C19* SNP with the lowest P-value in the basic model, increased from P =1.3 × 10^{-11} in the basic model to P = 1.5 × 10^{-4} in the extended model after adjusting for rs4086116. Thus in the extended model, this SNP no longer achieved genome-wide significance. However, rs1322179 and rs12767584, two other SNPs within *CYP2C19*, reached P-values of 10^{-6}, still showing some association with the outcome though not genome-wide significant. These SNPs just failed to be replicated in our second cohort with P-values = 0.06.

Meta-analysis. A meta-analysis with pooling of data from both cohorts was performed for the extended model with age, gender, BMI, target INR, rs10871454 (*VKORC1*) and rs4086116 (*CYP2C9*). P-values for the SNPs flanking *CYP2C18* improved from P <10^{-9} for the extended model within the index cohort to P< 10^{-12} in the meta-analysis (Table 3). There was also an improvement for rs2108622 within *CYP4F2* from P <10^{-8} to P <10^{-10}. Two more SNPs within *CYP4F2* became genome-wide significant in the meta-analysis. P-values for SNPs on chro-

Table 3 GWAS of acenocoumarol dosage adjusted for ***VKORC1*** and ***CYP2C9***, the 15 SNPs with lowest p-values (extended models)

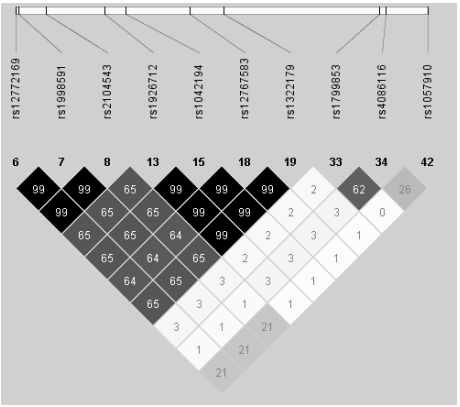
Chr	SNP (rs)	Gene	Base Pair position	RS-I cohort		RS-II cohort	Meta-analysis
				Extended model[a] P-value	Extended *CYP2C* model[b] P-value	Extended model[a] P-value	Extended model[a] P-value
10	1998591	CYP2C18	96397968	**$1.9*10^{-9}$**	$1.0*10^{-6}$	**$4.4*10^{-4}$**	**$4.9*10^{-12}$**
10	2104543	CYP2C18	96419961	**$2.5*10^{-9}$**	$1.2*10^{-6}$	**$4.1*10^{-4}$**	**$6.5*10^{-12}$**
10	12772169	CYP2C18	96395319	**$3.0*10^{-9}$**	$1.4*10^{-6}$	**$4.1*10^{-4}$**	**$7.7*10^{-12}$**
19	2108622	CYP4F2	15851431	**$2.0*10^{-8}$**	**$9.8*10^{-9}$**	**$3.0*10^{-3}$**	**$2.5*10^{-10}$**
19	2074901	CYP4F2	15858422	$1.9*10^{-7}$	$9.3*10^{-8}$	**$1.2*10^{-2}$**	**$8.3*10^{-9}$**
3	10935268	CNTN4	138930175	$7.7*10^{-7}$	$7.9*10^{-7}$	0.42	$8.18*10^{-7}$
3	9828150	CNTN4	2518302	$9.7*10^{-7}$	$4.5*10^{-7}$	0.56	$1.94*10^{-6}$
3	4571230	CNTN4	2513769	$1.3*10^{-6}$	$9.9*10^{-7}$	0.56	$9.1*10^{-6}$
10	1322179	CYP2C19	96565232	$1.6*10^{-6}$	$9.2*10^{-6}$	0.06	$2.6*10^{-7}$
10	12767583	CYP2C19	96537453	$1.6*10^{-6}$	$9.5*10^{-6}$	0.06	$2.8*10^{-7}$
10	1042194	CYP2C18	96485474	$1.6*10^{-6}$	$9.5*10^{-6}$	0.06	$2.7*10^{-7}$
10	1926712	CYP2C18	96467844	$1.7*10^{-6}$	$9.7*10^{-6}$	**0.04**	$2.0*10^{-7}$
10	2111995	No gene	107497352	$3.2*10^{-6}$	$6.6*10^{-6}$	0.77	$8.9*10^{-6}$
19	12610189	CYP4F2	1585422	$5.6*10^{-6}$	$3.3*10^{-6}$	**$4.2*10^{-4}$**	**$3.5*10^{-8}$**
11	1892889	No gene	90011495	$6.1*10^{-6}$	$7.9*10^{-6}$	0.9914	$2.7*10^{-5}$

Signficant results are printed in bold. Abbreviations: *CYP2C18* (Cytochrome P450 2C18), *CYP4F2* (Cytochrome P450 4F2), *CNTN4* (contactin 1), *CYP2C19* (Cytochrome P450 2C19).
a The extended model contained beside the clinical factors age, gender, BMI and target INR within unstandardized residuals also rs10871454 (VKORC1) and rs4086116 (CYP2C9) as independent covariates
b The extended *CYP2C* model contained the clinical factors age, gender, BMI and target INR within unstandardized residuals and also rs10871454 (*VKORC1*), rs4086116 (*CYP2C9*) and rs1057910 (*CYP2C9*3*) as independent covariates

mosome 3 remained at $P< 10^{-7}$ and failed to reach genome-wide significance in the meta-analysis. Further P-values for *CYP2C19* decreased from 10^{-6} with the extended model in the index population to 10^{-7} in meta-analysis, but were still not genome-wide significant.

Independency of signals within the *CYP2C* cluster. In order to elucidate in how far the influence on acenocoumarol dosage of the SNPs within *CYP2C18* were independent from *CYP2C9*, in an extended model on the *CYP2C* cluster we further adjusted for the effect of *CYP2C9**3 by adding rs1057910 as another independent co-variate to our extended model. We chose this SNP as linkage between rs4086116, the lowest scoring SNP for *CYP2C9* from our basic model, was reasonable adjusted with *CYP2C9**2 (r^2 =0.62) and poor with *CYP2C9**3 (r^2 = 0.26) (Fig. 7). Earlier it was shown that an association of rs4917639, being completely linked to rs4086116, could not fully be explained by *CYP2C9**3 [12]. Thus rs1057910, representing *CYP2C9*3,* was expected to provide extra information on CYP2C9 activity which could not fully be explained by rs4086116. The outcome of the analysis with the additional adjustment for *CYP2C9**3 showed that P-values of the three SNPs on chromosome 10 flanking *CYP2C18* increased from

Figure 7 Linkage disequilibrium of the within the CYP2C cluster on chromosome 10 within the RS-I cohort



rs12772169, rs1998591, rs2104543: flanking *CYP2C18*, top SNPs significant within the extended model.
rs1926712, rs1042194 within *CYP2C18*, just non-significant within the extended model. ($p>10^{-6}$)
rs12767583, rs1322179 within *CYP2C19*, just non-significant witin the extended model ($p>10^{-6}$)
rs1799853 for *CYP2C9*2*
rs4086116 within *Cyp2C9*, top SNP within the basic model
rs1057910 fpr *CYP2C9*3*

The LD-plot with =r^2 values is shown for different polymorphisms within *CYP2C18, CYP2C19* and *CYP2C9.*

10^{-9} up to 10^{-6}, which means that *CYP2C9**3 could partially but not completely explain variation on acenocoumarol dosage by SNPs flanking *CYP2C18* (Table 3). These three SNPs were in complete LD with each other (r^2= 0.99). Two other SNPs within *CYP2C18* were independent from *CYP2C9**3 and P-values in meta-analysis decreased from P =10^{-6} to P=10^{-7}, still not reaching genome-wide significance. These two SNPs were completely linked to each other (r^2 =0.99) and also in complete LD with the two lowest scoring SNPs within *CYP2C19*, rs1322179 and rs12767583 (Fig. 7).

Clinical implications

A multivariate model, including the clinical factors age, gender, BMI and target INR, but without any genotypic data explained 12.6% of acenocoumarol dosage variation (Table 4). The addition of rs9934438 (*VKORC1* C1173T) to this model dramatically increased the explained variance to 40.6% and the *CYP2C9**2/*3 polymorphism could add another 5.8%. Replacing the SNPs commonly used in literature for VKORC1 activity by rs10871454, the SNP with the lowest P-value from our results, slightly improved the percentage of explained variance from 46.4 to 46.6%. Replacing the commonly used combined *CYP2C9**2/*3 genotype by the SNP with the lowest P-value from our results, rs4086116, nearly explained the same amount of dosage variation than the combined genotype (0.3% less). A model including age, gender, BMI, target INR, rs10871454 for *VKORC1*, rs4086116 for *CYP2C9*, rs2108622 for *CYP4F2* and rs1998581 for *CYP2C18* could explain 48.8% of acenocoumarol dosage variation. Adding rs1057910 for the *CYP2C9**3 polymorphism only contributed an additional 0.1% to the explained variance. Each additional variant allele of the *CYP4F2* polymorphism and also each additional variant allele of the *CYP2C18* polymorphism increased the weekly acenocoumarol dosage by 1.2 mg. Genotype and allele frequencies of the top SNPs for *VKORC1, CYP2C9, CYP4F2* and *CYP2C18* were comparable between our study cohorts and to findings from literature (Table 5).

Table 4 Explained variance of acenocoumarol maintenance dosage in different models

Basic model	Added variables in different models	r^2adj^a
Age, gender, BMI, target INR		12.6%
Age, gender, BMI, target INR	rs9934438 (VKORC1 1173C>T)	40,6%
Age, gender, BMI, target INR	rs9934438 (*VKORC1 1173C>T*). rs1799853 and rs1057910 (combined *CYP2C9*2/*3* genotypes)	46.4%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results)combined rs1799853 and rs1057910 (combined CYP2C9*2/*3 genotypes)	46.6%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results), rs4086116 (Top SNP for *CYP2C9* in our results)	46.3%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results), rs4086116 (Top SNP for *CYP2C9* in our results), rs1057910 (*CYP2C9*3*)	46.7%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results), rs4086116 (Top SNP for *CYP2C9* in our results) rs1057910 (*CYP2C9*3*), rs2108622 (Top SNP for *CYP4F2* in our results)	48.0%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results), rs4086116 (Top SNP for *CYP2C9* in our results), rs1057910 (*CYP2C9*3*), rs2108622 (Top SNP for *CYP4F2* in our results), rs1998581 (Top SNP for *CYP2C18*)	48.9%
Age, gender, BMI, target INR	rs10871454 (Top SNP for *VKORC1* in our results), rs4086116 (Top SNP for *CYP2C9* in our results), rs2108622 (Top SNP for *CYP4F2* in our results), rs1998581 (Top SNP for *CYP2C18*)	48,8%

a r^2: adjusted for the variance of acenocoumarol dosage explained by the model

Table 5 Genotype and allele frequencies of the top SNPs for VKORC1, CYP2C9, CYP4F2 and CYP2C18

	RS-I cohort	RS-II cohort	From literature [13]
Chr 16: rs10871454 (%)(*VKORC1*)			
CC	527 (36.3)	114 (39.7)	169 (38.8)
CT	718 (49.5)	134 (46.7)	211 (48.4)
TT	206 (14.2)	39 (13.6)	56 (12.9)
Allelic frequency	C-allele 61.1% T-allele 38.9%		C-allele 63% T-allele 37%
Chr 10: rs4086116 (*CYP2C9*)			
CC	943 (65.0)	194 (67.6)	
CT	451 (31.1)	84 (29.3)	
TT	56 (3.9)	8 (2.8)	
Allelic frequency	C-allele 80.6% T-allele 19.4%		
Chr 10: rs1057910 (*CYP2C9*3*)*			
AA	1285 (88.6)	250 (87.1)	
AC	162 (11.2)	33 (11.5)	
CC	1 (0.1)	3 (1.0)	
Allelic frequency	A-allele 94.3% C-allele 5.7%		
Chr 19 rs2108622 (*CYP4F2*)			
CC	790 (54.4)	147 (51.2)	216 (49.6)
CT	539 (37.1)	223 (39.0)	183 (42.0)
TT	122 (8.4)	28 (9.8)	37 (8.4)
Allelic frequency	C-allele 73,0% T-allele 27,0%		C-allele 70.9% T-allele 29,1%
Chr 10 rs1998591 (*CYP2C18*)			
GG	897 (61.8)	168 (58.5)	
GA	487 (33.6)	104 (36.2)	
AA	66 (4.5)	15 (5.2)	
Allelic frequency	G -allele 79% A-allele 21%		

Discussion

In this large population-based cohort study, VKORC1 and CYP2C9 were the main factors regulating acenocoumarol-induced anticoagulation, in line with similar findings for warfarin. [32] In addition, polymorphisms in the *CYP4F2* gene had influence on acenocoumarol dosage. The genome-wide significant association for rs2108622 within *CYP4F2* and acenocoumarol dosage was replicated in our second cohort and was strengthened in the meta-analysis from our two study cohorts as P-values decreased and two more polymorphisms within this gene passed the genome-wide significant threshold of $P < 5 \times 10^{-8}$. Caldwell et al. [13] also found an association between the *CYP4F2* genotype and warfarin dosage. Until now the physiological role of CYP4F2 in coumarin response or vitamin K pathway is only partially elucidated. CYP4F2 hydroxylates the tocopherol phytyl side chain as the first step in the inactivation pathway of vitamin E.[34] As this side chain is quite similar to the side chains of the vitamins within the vitamin K group, CYP4F2 may also inactivate K vitamins by hydroxylation. Thus within the vitamin K cycle less substrate would be available for the activation of vitamin K-dependent coagulation factors. If variant alleles of rs2108622 decrease CYP4F2 activity as postulated before [13], this would prevent inactivation of vitamin K and thus explain the need for higher acenocoumarol dosages with ~1 mg/week more per additional variant allele. Adding rs2108622 to a model of clinical factors, *VKORC1* and *CYP2C9* genotype information increased r^2adj for acenocoumarol dosage variation by 1.3%. This is in line with the results of Caldwell et al [13] for warfarin where this SNP contributed with 2% to explanation of warfarin dosage variability.

In our GWAS of acenocoumarol dosage, rs10871454 had the lowest P-value. This SNP was located on chromosome 16, close to *VKORC1*. Cooper et al. [32] have previously found this SNP to be most strongly associated in a GWAS with warfarin dosage. Due to more subjects in our study, the P-value of this SNP in our study was 2.0×10^{-123} compared with a P-value of 6.2×10^{-13} in the earlier study. The clinical implications of this SNP are unknown. As it is situated within another gene, the Syntaxin 4 gene, it is not coding for VKORC1 and thus cannot directly alter the structure of this protein. However, such polymorphisms might lower the intrahepatic mRNA expression for this enzyme, resulting in decreased enzyme activity which reduces the amount of drug target in the liver and leads to lower dosage requirement of coumarins.[33] The effect of rs10871454 may be through LD with a putative functional SNP within *VKORC1*. In our study population, rs10871454 was in complete LD (r^2 =0.99) with rs9934438 (*VKORC1* 1173C>T), one of the five single segregating SNPs that were able to explain all variation in warfarin dose caused by *VKORC1*.[7, 33] Earlier studies suggested rs9934438 or rs8050894 as the possible putative functional SNP responsible for the association between *VKORC1* and coumarin dosage, as within the five representative *VKORC1* SNPs, being in complete LD with each other, these two SNPs were the ones that remained conserved across species.[5] However, clinical implications may be only small as rs10871454 contributed only 0.2% more to the explained variance in acenocoumarol maintenance dosage than rs9934439.

Besides chromosome 16, only SNPs on chromosome 10 reached genome-wide significance. This chromosome contains within a region of high LD a cluster of the following *CYP2C* genes: *CYP2C8, CYP2C9, CYP2C18* and *CYP2C19*. Most strongly associated with acenocoumarol dosage were SNPs within the *CYP2C9* gene, rs4086116 reaching the lowest P-value with $P = 3.29 \times 10^{-24}$. This SNP was in complete LD with SNP rs4917639 ($r^2 = 1.0$) which had been shown previously to be significantly associated to warfarin dosage.[12] This SNP was able to explain nearly as much of dosage variation as the combined *CYP2C9**2/*3 genotype (only 0.3% less). Besides *CYP2C9*, only polymorphisms flanking *CYP2C18* showed associations with acenocoumarol maintenance dosage that could not be fully explained by *CYP2C9* polymorphisms. Possible associations of polymorphisms in the *CYP2C19* gene could be explained through complete LD of these SNPs with polymorphisms in *CYP2C18.* For loci in the *CYP2C8* gene, no association with acenocoumarol dosage could be demonstrated. In an earlier study, associations of *CYP2C18* and *CYP2C19* with warfarin dosage were fully explained by *CYP2C9**2 and/or *3 polymorphisms.[12] The independence of *CYP2C18* from *CYP2C9* polymorphisms in our study may be due to the fact that pharmacokinetics by CYP2C enzymes differs between warfarin and acenocoumarol. Adding rs1998591 as an indicator for variance in CYP2C18 activity to our egression model increased the r^2adj of acenocoumarol dosage variance by 1.2%. In comparison, adding the *CYP2C9**3 SNP (rs1057910) to a model already including rs4086116 for *CYP2C9* polymorphisms increased the percentage of explained acenocoumarol dosage variation by only 0.1%.

The density and coverage of the polymorphism set used (HumanHap 550K) is substantial and represents ~90% of the common SNP variation in Caucasians as determined by the HapMap. Our measure of maintenance dosage was an incidental outcome as the dosage at the end of 6 weeks of the initiation period and might have been influenced by coincidental factors such as diet or concomitant use of substrates or inhibitors of metabolic enzymes such as CYP2C9. However as diet and prescription of co-medication are independent from genotypes, this should lead to non-random misclassification and rather weaken than inflate associations found. Nevertheless, we repeated our analysis in the index population for outcomes adjusted for vitamin K intake and also for current use of CYP2C9 co-medication. Both subanalyses did not change our results. Analysis with the first INR after standard initial acenocoumarol dosage confirmed our results for the *VKORC1* polymorphisms as contributing most to interindividual variation in response to initial acenocoumarol treatment.

To our knowledge, this is the first GWAS for response to acenocoumarol dosage. We consider the chance of bias and confounding in this retrospective study as negligible. First, the Rotterdam study is a prospective cohort study and the regional anticoagulation clinic covered a complete area of more than one million inhabitants in the Rotterdam region. Consequently, everyone who is treated with a coumarin anticoagulant as an outpatient will be registered as such and selection bias is highly unlikely, confirmed by the fact that in our study population genotypes were within Hardy Weinberg equilibrium (HWE). Also allele frequen-

cies of the variant alleles were comparable to results for the same SNPs in other Caucasian populations.[13] Furthermore, acenocoumarol dosage and INR are well-defined phenotypes and our results were biologically plausible. We confirmed earlier associations with warfarin dosage for rs10871454 related to *VKORC1* [32] and rs2108622 within *CYP4F2* [13]. One of the strengths of our study lies in the high numbers of patients included. Thus power to detect possible associations for new genes independently from *VKORC1* and *CYP2C9* was considerably larger than in earlier studies.[32] However, we did not detect further independent associations with acenocoumarol dosage besides *VKORC1* and *CYP2C9* for other genes than *CYP4F2* and *CYP2C18*.

Conclusion

Our findings confirmed that acenocoumarol maintenance dosage mainly depends on polymorphisms in the *VKORC1* and *CYP2C9* genes. Independent from these two genes, only DNA variants in the *CYP4F2* gene had a small additional influence on acenocoumarol dosage. From the *CYP2C* gene cluster on chromosome 10, only polymorphisms in the *CYP2C18* gene added some extra information besides *CYP2C9* polymorphisms on acenocoumarol dosage variation.

Abbreviations

Genome-wide Association Study; GWAS
Single Nucleotide Polymorphisms: SNPs
Vitamin K epoxide reductase complex subunit 1: *VKORC1*
International Normalized Ratio: INR
Body Mass Index: BMI
Rotterdam Study baseline cohort: RS-I
Rotterdam Study first extended cohort: RS-II
Hardy Weinberg Equilibrium: HWE
Cytochrome P450 2C9: *CYP2C9*
Cytochrome P450 4F2: *CYP4F2*
Cytochrome P450 2C8: *CYP2C8*
Cytochrome P450 2C9: *CYP2C9*
Cytochrome P450 2C18: *CYP2C18*
Cytochrome P450 2C19: *CYP2C19*
Syntaxin 4 A – placental gene: *STX4A gene*
Contactin 1: *CNTN4*

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Chapter 3.3

Dependency of phenprocoumon dosage on polymorphisms in the *VKORC1*, *CYP2C9* and *CYP4F2* genes

Abstract

Genome-wide association studies (GWAS) on warfarin and acenocoumarol showed that interindividual dosage variation mainly is associated with single nucleotide polymorphisms (SNPs) in *VKORC1* and to a lesser extent with *CYP2C9* and *CYP4F2*. For phenprocoumon dosage also the genes encoding *CYP3A4* and *ApoE* might play a role. Our objective was to asses the association between common genetic variants within *VKORC1*, *CYP2C9*, *CYP4F2*, *CYP3A4* and *ApoE* and phenprocoumon maintenance dosage, as well as to identify novel signals using GWAS. We selected all subjects from the Rotterdam Study who were treated with phenprocoumon. For each SNP, we tested the association between the above-mentioned genotypes and age-, sex-, body mass index- and target international normalized ratio (INR) adjusted-phenprocoumon maintenance dosage. Within our study population (N=244), *VKORC1*, *CYP2C9*, *CYP4F2* genotypes together explained 46% of phenprocoumon maintenance dosage variation. Each additional *VKORC1* variant allele reduced phenprocoumon maintenance dosage by 4.8 mg/week ($p<0.0001$) and each additional *CYP2C9* variant allele by 2.2 mg/week ($p=0.002$). Each additional variant allele of *CYP4F2* increased phenprocoumon dosage by 1.5 mg/week ($p=0.022$). Variant alleles of *CYP3A41*B* and of *ApoE* showed no association with phenprocoumon dosage. Genome wide significant SNPs were all related to *VKORC1* activity. Best associated were two SNPs in complete linkage disequilibrium with each other and with SNPs within *VKORC1:* rs10871454 (Syntaxin 4A (*STX4A*)) and rs11150604 (*ZNF646*), each with a P-value of 2.1×10^{-22}. Each reduced weekly phenprocoumon maintenance dosage by 4.9 mg per variant allele. In conclusion, similar to earlier findings with warfarin and acenocoumarol, phenprocoumon maintenance dosage depended on polymorphisms in the *VKORC1* gene. *CYP2C9* and *CYP4F2* were of modest relevance.

Introduction

Coumarin anticoagulants are first choice in treatment and prevention of thromboembolic diseases.[1] Clinical management of anticoagulation with coumarins is difficult, as a target range has to be achieved by drugs with a narrow therapeutic index and high intra- and interindividual variability in pharmacokinetic and pharmacodynamic response. The importance of maintaining coumarin users within the therapeutic range is driven by the aim of preventing thromboembolic events as well as by the necessity to minimize the risk of serious bleeding, the main manifestation of coumarin toxicity. Despite management of coumarin use by regular measurements of international normalized ratio (INR) through anticoagulation clinics in the Netherlands, these drugs still are a leading cause of preventable hospital admissions.[2, 3] Genetic variation in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 2C9 (*CYP2C9*) genes in combination with age, sex and body mass index (BMI) has been shown to account for 30-50% of the variability in dosage of warfarin and acenocoumarol.[4-11] According to genome-wide association studies (GWAS) of warfarin and acenocoumarol, dosage variation was mainly associated with certain single nucleotide polymorphisms (SNPs)in linkage disequilibrium with SNPs within *VKORC1* and to a lesser extent in *CYP2C9* and *CYP4F2*.[12-14] Genetic variation for *VKORC1* resulted in 28% of warfarin- or acenocoumarol dosage variation between subjects, variant alleles in *CYP2C9* variation explained 9% for warfarin and 6% for acenocoumarol maintenance dosge and a *CYP4F2* polymorphism counted for 1% of coumarin dosage variation. For phenprocoumon, used frequently in many European countries, fewer candicate gene studies have been conducted and a GWAS has not been performed so far. Some studies were suggestive of associations between phenprocoumon maintenance dosage or bleedings and *CYP2C9* polymorhisms [15-20] and one study showed an increased bleeding risk with increasing number of *VKORC1* variant alleles [21]. Two studies investigated *VKORC1* and *CYP2C9* polymorphisms together and found that polymorphisms in both genes influenced anticoagulation therapy with phenprocoumon.[22, 23] The metabolism of phenprocoumon however, is different from the other coumarins and therefore influences of CYP-isoenzymes on phenprocoumon maintenance dosage might differ from those already shown for warfarin and acenocoumarol. [18, 24, 25] The effect of *CYP2C9* should be smaller, as 40% of phenprocoumon is eliminated unmetabolized in contrast to warfarin and acenocoumarol which are fully metabolized by CYP enzymes.[26] Until now, data for the influence of *CYP4F2* on the activity of phenprocoumon are lacking. Effects of *CYP4F2* polymorphisms on phenprocoumon dosage might be similar to those on warfarin and acenocoumarol, as this enzyme seems to reduce the amount of available vitamine K.[27] Genotypes of the polymorphic protein Apolipoprotein E (*ApoE*) were also shown to affect acenocoumarol dosage. These gentoypes are defined by three alleles ε2, ε3 and ε4 at a single gene locus on chromosome 19.[28] Since they affect plasma vitamin K levels they are also likely to influence phenprocoumon dosage. Phenprocoumon

hydroxylation has been shown in vitro to depend, besides CYP2C9, on CYP3A4.[18, 25] The G allele of the polymorphism*CYP3A4**1B (-392A>G), rs2740574, located in the promotor region of the *CYP3A4* gene, was associated with enhanced CYP3A4 expression due to reduced binding of a transcriptional repressor.[29]

The objective of our study was to assess whether and to what extent common genetic variants within *VKORC1*, *CYP2C9*, *CYP4F2*, *ApoE* and *CYP3A4* modify the dosage requirement of phenprocoumon. As a second objective we performed a hypothesis-free genome wide association study to find novel associations with phenprocoumon maintenance dosage as the outcome of interest.

Methods

Setting and inclusion criteria

We selected all subjects from the first two cohorts of the Rotterdam Study (RS-I and RS-II). The rationale and design of the Rotterdam Study have been described elsewhere.[30-32] In brief, the Rotterdam Study is a prospective population-based cohort study, consisting of 98% Caucasians and designed to study neurological, cardiovascular, locomotor and ophthalmologic diseases in a population of people of 55 years and older. The RS-I cohort consisted of 7,983 subjects (response 78%) and the RS-II cohort of 3,011 participants (response 67%). The RS-I cohort had baseline examinations during 1990 – 1993 with completion of standardized questionnaires, sampling of blood and isolation of DNA. The RS-II was formed as an independent cohort in 1999 with baseline examinations between 2000 and 2001. A regional anticoagulation clinic monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. This clinic covers the patients from the RS-I cohort as well as those from the RS-II cohort. From this clinic, since 1984, all data on dosing, laboratory and clinical data, including data on bleeding complications are fully computerized. We could retrieve data for the subjects of the first two cohorts of the Rotterdam Study as of 1985. This was permitted by the Medical ethical Committee of the Erasmus Medical Center as all participants gave full written consnt. The physician who treats the patient makes the choice of anticoagulant. Prothrombin times are monitored every 1-6 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 computerized dose calculations.

Our study population consisted of all patients of the RS-I and RS-II cohorts in whom genotyping or imputation was succesful and who started with phenprocoumon in the study period between 30th October 1985 and 9th September 2009, using it consecutively for at least 42 days. From the 5,974 subjects of the RS-I cohort who were genotyped and had passed quality control, 202 (3%) started phenprocoumon therapy in our study period between 30th October 1985 and 9th September 2009 and continued therapy for at least 42 days. From the

2,157 participants of the RS-II cohort with succesfull genotyping, 42 subjects (2%) used phenprocoumon during the study period for at least 42 days. Consequently, the study population comprised of 244 subjects. For the GWAS, we used the RS-I cohort with 202 patients as index cohort and the 42 patients from the RS-II cohort for validation of novel signals.

Genotyping

From all RS participants those with available DNA were genotyped using Illumina Infinium II HumanHap BeadChips, Illumina Inc, San Diego, Los Angelos, USA, at the Department of Internal Medicine, Erasmus Medical Center following manufacturer's protocols. RS-I participants (n=6,449) were genotyped with 550k (V.3) single and duo chips, while RS-II participants (n=2,516) were genotyped with 550k (V.3) duo and 610k Quad chips. Genotype calling was performed in RS-I using BeadStudio software (version 0.3.10.14) and GenomesStudio in RS-II. Participants with call rates <97.5%, excess autosomal heterozygosity, sex mismatch or outlying identity-by-state clustering estimates were excluded. After quality control, 5,974 RS-I participants and 2,157 RS-II participants remained with complete data on genotyping.[33] For imputation 512 349 autosomal SNPs in RS-I and 466 389 autosomal SNPs in RS-II were used after exclusions for call rates less than 98%, Hardy-Weinberg equilibrium P value of less than 10 – 6, and minor allele frequency (MAF) of less than 1%, in MACH (softwarepackage within METAL) (version 1.00.15 for RS-I and 1.00.16 for RS-II) with reference to the 2 543 886 SNPs of the HapMap CEU (release 22, build 36, http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/)[34, 35] The SNPs coding for the *ApoE* haplotypes, rs429358 and rs7412, were not available form the imputed database. Therefore we used *ApoE* genotypes available for the RS-I cohort as described before.[28]

Phenotype definition

Physicians aim for a therapeutic target INR by titrating phenprocoumon dosage. The quantitative outcome for our study was the phenprocoumon maintenance dosage (mg/week) given to a patient after 42 days of continuous use. Steady state of a drug is usually achieved within 5-7 half-lives of drug elimination. For phenprocoumon with a half-life of 160 hours (6,7 days), steady state would be reached after a treatment period of at least 5 weeks.[36]

Statistical analysis

Candidate gene study

For the association of variant alleles in the genes *VKORC1*, *CYP2C9* and *CYP4F2*, we used the following SNPs: rs10871454, located 60 kb 5' of *VKORC1*, rs4086116 for *CYP2C9* and rs2108622 for *CYP4F2*. These SNPs had scored the lowest p-values within these genes in our earlier GWAS with acenocoumarol and were available for both cohorts.[14] Furthermore rs10871454 was in complete linkage disequilibrium (LD, $r^2 = 0.99$) with rs9934438 (VKORC1 113C>T), one of the five single segregating SNPs that were able to explain all variation in warfarin dose caused by

VKORC1.[6, 37] This tag SNP for *CYP2C9*, *rs*4086116, was in complete LD with rs4917639, a SNP shown to be significantly associated to warfarin dosage and to be in perfect LD (r^2=1) with a composite minor allele formed by aggregating *CYP2C9**2 and *3.[11] The genetic variance of *CYP3A4**1B (-392A>G) was represented by rs2740574[29] and for *ApoE* we used the three alleles ε2, ε3 and ε4 at a single gene locus on chromosome 19.[28] Phenprocoumon maintenance dosage was compared in *VKORC1*, *CYP2C9*, *CYP4F2*, *CYP3A4* and *ApoE* genotypes in allele effect models with linear regression analysis adjusted for age, sex, target INR, BMI and cohort. Target INR was included into our model in order to adjust for the necessity of higher dosages due to intended therapeutic INR values for certain indications. BMI was defined as (kg/m^2) and missing values were imputed with a regression model consisting of maintenance dosage as outcome with age, sex and target INR as variables. In order to adjust for differences between the two cohorts and the way of genotyping, we also adjusted for cohort origin. Interaction between variables was tested with interaction terms.

GWAS

For the GWAS the overall strategy involved linear regression analysis for the genotype–phenotype association of 2.5 million markers. SNPs with a Minor Allele Frequency (MAF) < 0.05 were excluded from the analysis. We chose a MAF of 0.05 as a threshold in order to increase the specificity of positive findings. We considered results significant at $p < 5*10^{-8}$, based on estimation of the multiple testing burden for genome-wide association studies of nearly all common variants in the human genome of European ancestry individuals for a target genome-wide P<0.05.[38] For replication of the specific SNPs associated with phenprocoumon dosage within the index cohort, in the replication cohort a p-value < 0.05 was considered significant. This was done with a basic model for phenprocoumon maintenance dosage as the outcome, adjusted for age, sex and BMI and the patient's target INR. We performed a meta-analysis with the results from the basic model for the RS-I- and RS-II-cohort to strengthen our findings from the index cohort. In order to detect SNPs within other genes independently associated with phenprocoumon dosage and different from those detected in the basic model, we repeated GWAS in an extended model further adjusting for influences of the genes having a genome wide significant association with the outcome in the basic model as done previously in the GWAS of warfarin [13] and on acenocoumarol [14]. In the extended model these SNPs were to be taken as independent co-variables that were found by the basic model to be the strongest associated SNPs within the gene.

Software

Multivariate linear regression analysis was performed with SPSS software, version 15.0. (IBM corp., Chicago, USA) For the genome-wide association analysis we used ProbABEL (http://mga.bionet.nsc.ru/Byurii/ABEL/ [39]). The meta-analysis was performed using METAL (http://www.sph.umich.edu/csg/abecasis/Metal/index.html).

Results

Cohort characteristics

The 244 subjects of our study population were on avarage 75 years old and 62% were male (table1). For BMI, values for 5 persons (2%) had to be imputed. The mean BMI of the study population was 26.8. In our study population, phenprocoumon was prescribed with a very low target range (2.0–2.5) for short-term prophylactic treatment, with a low target INR range (2.5–3.5) also in patients for short-term prophylactic treatment, for deep venous thrombosis, pulmonary embolism, atrial fibrillation and cerebral ischemia and with medium target INR range (3.0–4.0) in patients with atrial fibrillation, myocardial infarction, vascular surgery, stroke, transient ischemic attacks and periphery artery disease. The majority (68%) used phenprocoumon with a medium INR target range. The mean weekly dosage of phenprocoumon was 13.8 mg/week with a range from 0.77–57.8 mg/week. The 25, 50 and 75 percentiles were 9.0, 12.7 and 18.0 mg/week respectively.

Table 1 Description of the study population with phenprocoumon users

	Number of patients (%)
Patients within RS-cohorts	244
RS-I	202
RS-II	42
Age in years, average (SD)	75.2 (9.2)
Sex (%)	
Male	151 (62)
Female	93 (38)
Body Mass Index (BMI), average (SD)	26.8 (3.7)
Number of patients within a target INR-level (%):	
Very low: 2.0 – 2.5	4 (2)
Low: 2.5 – 3.5	74 (30)
Medium: 3.0 – 4.0	166 (68)
Mean weekly dosage [mg/week] at the end of the initiation period of 42 days (range)	13.8 (0.77 – 57.8)

Genotype-phenotype associations and explained variance

Each additional *VKORC1* variant allele reduced phenprocoumon maintenance dosage by 4.8 mg/week ($p<0.0001$) and each additional *CYP2C9* variant allele by 2.2 mg/week ($p=0.002$). Each additional variant allele of *CYP4F2* increased phenprocoumon dosage by 1.5 mg/week ($p=0.022$) (table 2). There was no significant multiplicative interaction of effect measures between the variant alleles of these genes ($p=0.70$ for an interaction between polymorphisms in *VKORC1* and *CYP2C9*, $p=0.60$ for *VKORC1* and *CYP4F2* and $p=0.38$ for interaction between *CYP2C9* and *CYP3A4*). Variant alleles of *CYP3A41**B and of *ApoE* showed no association with phenprocoumon dosage. There was a borderline significant trend for each additional allele ε3 and ε4 allele of *ApoE* with decreasing phenprocoumon dosage ($p=0.04$), however with modest effect (beta = -0.005 mg/week). We found additive effects of the variant alleles of *CYP2C9* and *CYP4F2* on *VKORC1* (see figure 1 for *VKORC1* and *CYP4F2*).

Table 2 Phenprocoumon maintenance dosage by genotype

Genotype	Number of patients (%)	Median maintenance dosage in mg/week (SD)	Difference with wild-type genotype in mg/week[b,c]	p value
STX4A[a]: rs10871454	244 (100)		Trend per allele: **-4.78 (-5.68 to -3.88)**	<0.0001
CC	90 (36.9)	17.2 (7.48)	-	
CT	101 (41.4)	12.7 (5.35)	**-4.51 (-6.06 to – 2.97)**	<0.0001
CT	53 (21.7)	8.3 (4.61)	**-9.62 (-11.46 to -7.78)**	<0.0001
Allelic frequency	C-allele 57.6%, T-allele 42.4%			
CYP2C9: rs4086116	244 (100)		Trend per allele: **-2.19 (-3.59 to -0.78)**	0.002
CC	162 (66.4)	13.5 (7.13)	-	
CT	71 (29.1)	11.3 (6.17)	**-3.13 (-4.92 to -1.35)**	0.001
TT	11 (4.5)	12.0 (8.72)	-2.08 (-5.97 to 1.80)	0.291
Allelic frequency	C-allele 80.9%, T-allele 19.1%			
CYP4F2: rs2108622	244 (100)		Trend per allele: **1.47 (0.21 to 2.74)**	0.023
CC	119 (48.8)	12.7 (7.13)	-	
CT	104 (42.6)	12.7 (6.47)	0.62 (-1.07 to 2.32)	0.470
TT	21 (8.6)	18.0 (7.71)	**4.18 (1.17 to 7.12)**	0.007
Allelic frequency)	C-allele 70.1%, T-allele 29.9%			
*CYP3A4*1B:* rs2740574	242 (100)		Trend per allele: 1.54 (-1.59 to 4.68)	0.33
AA	224 (92.6)	12.7 (7.11)	-	
AG	18 (7.4)	14.3 (5.82)	1.54 (-1.59 to 4.68)	0.33
GG	0 (0)	-	-	-
Allelic frequency)	A-allele 96.3%, G-allele 3.7%			
ApoE	194 (100)		Trend per allele: **-0.01 (-0.01 to 0.00)**	0.04
ε2/ε2	2 (1.0)	11.2 (9.55)	-0.46 (-10.2 to 9.15)	0.93
ε2/ε3	28 (14.4)	13.1 (5.42)	-0.05 (-2.93 to 2.84)	0.97
ε3/ε3	106 (54.6)	12.0 (8.38)	-	-
ε3/ε4	51 (26.3)	13.5 (5.62)	-0.07 (-2.34 to 2.21)	0.95
ε2/ε4	4 (2.1)	11.3 (5.31)	-2.96 (-9.82 to 3.89)	0.40
ε4/ε4	3 (1.5)	20.2 (6.40)	3.36 (-4.57 to 11.3)	0.40
ε2 carriers	30 (15.5)	13.5 (5.56)	-0.26 (-9.78 to 9.26)	0.96
ε4 carriers	54 (27.8)	13.81(5.87)	0.22 (-1.93 to 2.34)	0.85
Allelic frequency	ε 2 allele 9.3%, ε3 allele 75%, ε4 allele 15.7%			

a *STX4A* is closely linked to rs9934438, possibly one of the putative funictional SNPs within *VKORC1*.
b Linear regression with phenprocoumon maintenance dosage, adjusted for age, sex, BMI, target INR and cohort.
c Significant values are printed in bold. Threshold for the p-values of the association of the allelic effect model with dosage was 0.05, for p-values of the HWE a treshold of 10^{-6} was taken for significancy, see Methods section.
SD = Standard deviation. P-values of Hardy-Weinberg Equilibrium (HWE): for STX4A = 0.02, for CYP2C9 = 0.37; for CYP4F2 = 0.80; for CYP3A4*1B = 0.55; for ApoE = 0.58.

Figure 1 VKORC1 and CYP4F2 genotypes associated with phenprocoumon maintenance dosage [mg/week]

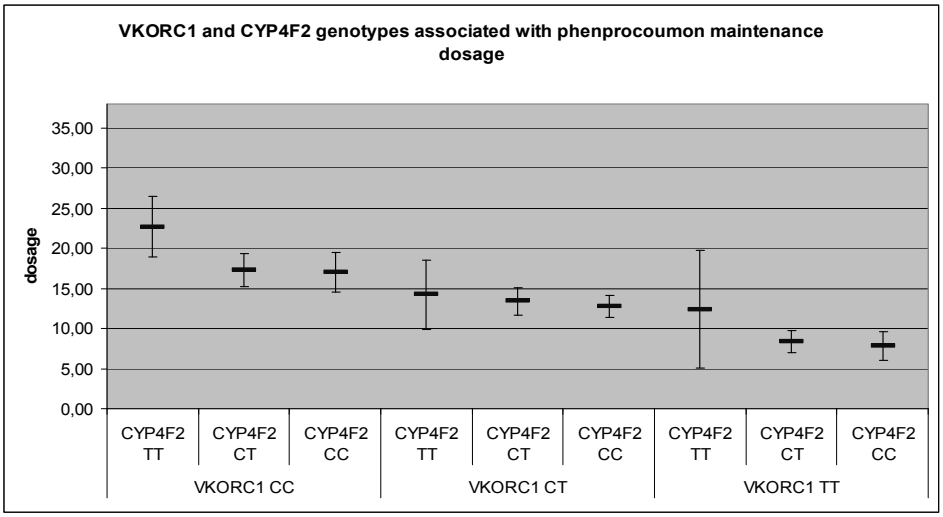


Table 3 Explained phenotypic variance of phenprocoumon maintenance dosage

Basic model	Added variables	r^2 adj [a]
Age, sex, BMI, target INR		16.1%
Age, sex, BMI, target INR	rs10871454 (*STX4A*[b])	42.2%
Age, sex, BMI, target INR	rs10871454 (*STX4A*) and rs4086116 (*CYP2C9*)	45.1%
Age, sex, BMI, target INR	rs10871454 (*STX4A*), rs4086116 (*CYP2C9*) and rs2108622 (*CYP4F2*)	46.0%

a Adjusted variance of phenprocoumon dosage explained by the model. The analysis is also adjusted for the cohort.
b *STX4A* is closely linked to rs9934438, possibly one of the putative funictional SNPs within *VKORC1*.

Within our study cohort the multivariate basic model including age, sex, BMI and target INR explained 16.1% of phenprocoumon maintenance dosage (table 3). Adding rs10871454 (*VKORC1*) to this model markedly increased the explained variance to 42.2%. SNP rs4086116 (*CYP2C9*) could add 2.9% and rs2108622 (*CYP4F2*) contributed an additional 0.9%. A final model of the basic variables together with genetic variation of *VKORC1, CYP2C9* and *CYP4F2* explained 46.0% of phenprocoumon maintenance dosage variation.

GWAS

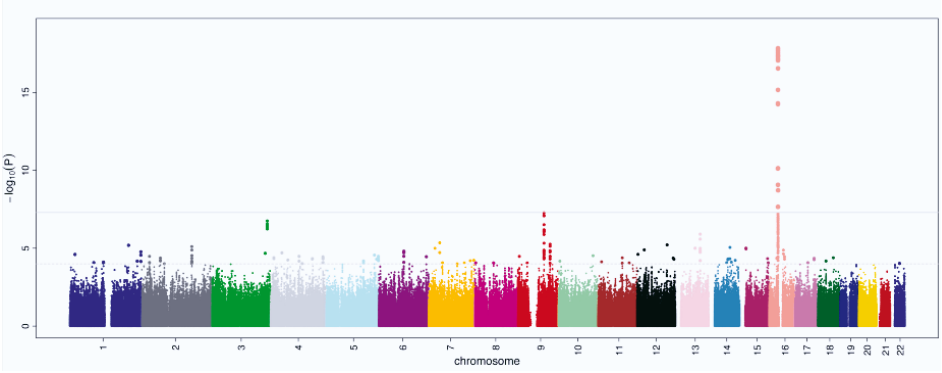
In the RS-I cohort, 32 SNPs with a MAF>0.05 were in genome wide significant association with phenprocoumon maintenance dosage, adjusted for age, sex, BMI and target INR. They were all located on chromosome 16 between position 30432177 and 31241737. The *VKORC1* gene is located at 31102175 - 31106276. Strongest association was observed for rs10871454, with 4.9 mg/week decrease of phenprocoumon dosage per minor allele copy (MAF= 0.39, position 30955580, $P=1.4*10^{-18}$, table 4, figure 2). Similarly associated was rs11150604. Per

Table 4 GWAS of phenprocoumon maintenance dosage, basic model

Chr	SNP	Gene	Base Pair position	RS-I cohort		RS-II cohort	Meta-analysis, model
				Beta[a]	P-value	P-value	P-value
16	rs11150604	ZNF646	30944521	-4.9	**$1.4*10^{-18}$**	**$2,5*10^{-3}$**	**$2,1*10^{-22}$**
16	rs10871454	*STX4Ab*	30955580	-4.9	**$1.4*10^{-18}$**	**$2,5*10^{-3}$**	**$2,1*10^{-22}$**
9	rs1980889	*CKS2*	91089065	-5.6	$5.3*10^{-8}$	0.39	$4.9*10^{-5}$
9	rs746357	*SHC3*	91027380	-5.7	$7.9*10^{-8}$	0.71	$2.3*10^{-4}$

Signficant results are printed in bold. The basic model: adjusted for age, sex, BMI and target INR.
The two SNPs with the lowest P-values each within chromosome 16 and chromosome 9 are reported. For all 32 genome wide significant SNPs from the basic model, we supply an online supplement for complete overview.
a dosage change of phenprocoumon in mg/week per additional variant allele
b *STX4A* is located 60 kb 5 of *VKORC1* and closely linked to rs9934438, possibly one of the putative functional SNPs within *VKORC1*.
Gene abbreviations: *ZNF64* (Zinc finger protein 646), *STX4A* (Syntaxin 4 A), *CKS2* (CDC28 protein kinase regulatory subunit 2), *SHC3* (SHC (Src homology 2 domain containing) transforming protein 3).

Figure 2 Genome wide p-values for phenprocoumon dosage association in the RS-I-population , adjusted for age, sex, BMI and target INR (MAF>0.05)



additional minor allele copy the phenprocoumon maintenance dosage decreased with 4.9 mg/week (MAF = 0.39, position 30944521, $P=1.4*10^{-18}$). These two SNPs were in complete linkage disequilibrium with each other ($r^2=1.00$) and signals were replicated in the RS-II cohort (p-value <0.05). In meta-analysis P-values of these decreased further ($p=2.1*10^{-22}$). On chromosome 9 we found four borderline significant signals: rs1980889 (MAF=0.11, position 91089065, $P = 5.3*10^{-8}$, table 4), on the Cyclin-dependent kinases regulatory subunit 2 (*CKS2*) gene, decreased phenprocoumon maintenance dosage with 5.6 mg/week; the other three SNPs n chromosome 9 with a p-value of $8*10^{-8}$, were all on the *SHC* (Src homology 2 domain containing) transforming protein 3 (SHC3). However, these four SNPs on chromosom 9 were not replicated in the RS-II cohort. In meta-analysis their p-values increased up to 10^{-4}. In the extended GWAS-model, we included the SNP rs1087145 as independent covariable beside age, sex, BMI and target INR. From this analysis, no SNPs passed the threshold for genome-wide significance for $p<5*10^{-8}$. However there were SNPs with p-values $<10^{-7}$, all on

chromosome 9 in the genes *CKS2* (catalytical subunit of the cyclin dependent kinase), *SHC3* (Src homology 2 domain containing transfroming protein 3)and *IL33*(Interleukin 33).

Discussion

Our study confirms that apart from therapeutic considerations such as target INR, variation in phenprocoumon maintenance dosage mainly depends on a *VKORC1* polymorphism, similar to warfarin and acenocoumarol. VKORC1, the target of coumarins, is the essential enzyme in regenerating vitamin K which is oxidated when contributing to activation of coagulation factors. Subjects with variant alleles of *VKORC1* express less VKORC1 enzyme, have reduced ability to regenerate vitamin K and are less able to activate coagulation factors. Consequently, a lower dosage of coumarins is needed to reduce coagulation activity within the therapeutic target range. The GWAS brought up rs10871454 (Syntaxin 4 A (*STX4A*)) and rs1150604 (Zinc finger protein 646 (*ZNF646*)) as variants with the strongest association with phenprocoumon dosage. Two GWAS were earlier performed on warfarin and acenocoumarol.[13, 14] In both GWAS, the SNP rs10871454 was the one strongest associated with maintenance dosage as it was the case in our GWAS on phenprocoumon. Within our imputed dataset, rs1150604 was as strongly associated. This SNP is in complete linkage disequlibrium with rs10871454 and their effect may be through LD with a putative functional SNP within *VKORC1*.[6, 37] In our study population a *VKORC1* polymorphism explained 26% of phenprocoumon dosage variation between patients and each variant allele decreased the weekly phenprocoumon dosage by nearly 5 mg. Thus, the influence of rs10871454 on phenprocoumon is similar to that on the other coumarins, explaining approximately 28% of the warfarin and acenocoumarol dosage variation [12, 14]. There were borderline significant signals in the *SHC3*, *CKS9* and *IL33* genes. These genes code for proteins not known for specific biological functions within coagulation or interfering with phenprocoumon effectiveness. The *CKS2* gene encodes a protein that binds to the catalytic subunit of the cyclin dependent kinases and is essential for their biological function. [40] *SHC3* codes for the Src homology 2 domain containing transforming protein 3 which is involved in the signal transduction pathways in cortical neurons.[40] The *IL33* defines the interleukin 33 protein that induces T helper cytokines.[40]As numbers in the replication cohort were too low, we could not significantly replicate these findings nor could we strengthen the association in the meta-analysis. Within larger study populations these signals should be further elucidated. Repeating GWAS after adjustment for *VKORC1*, revealed no further genome-wide significant signals. This may be due to low numbers in the index cohort as the association with *CYP4F2* which we earlier showed with acenocoumarol required nearly 1,500 subjects to be able to pass the genome wide significance threshold. From the studies with phenprocoumon conducted so far, only one had a higher numbers of users (N=281) [23] than our study and other studies included less than 100 patients.[16]

In the GWAS we also did not detect an association of phenprocoumon maintenance dosage with *CYP2C9* polymorphisms. This may be explained by lack of power as well as by a weaker dependency of phenprocoumon kinetics on this enzyme than warfarin and acenocoumarol.[24] As a candidate gene, *CYP2C9* polymorphisms could explain an additional 3% of interindividual variation of phenprocoumon maintenance dosage in a model with age, sex, BMI, target INR and a *VKORC1* polymorphism. As expected, the influence of *CYP2C9* polymorphisms on phenprocoumon dosage was lower than on warfarin dosage (12% of explained dosage variance [12]) and on acenocoumarol (6% of explained dosage variance [14]). Each *CYP2C9* variant allele reduced weekly phenprocoumon dosage by 2.2 mg. CYP2C9 catalyses the biotransformation of S-warfarin, R- and S-acenocoumarol and 4', 6 and 7 hydroxylation of S- and R-phenprocoumon.[17] Patients with *CYP2C9* polymorphisms metabolize coumarins slower which requires a lower maintenance dosage to stay within the therapeutic window. Associations of *CYP2C9* polymorphisms with warfarin and acenocoumarol dosage have been shown convincingly in various studies.[15, 41, 42] However, unlike warfarin and acenocoumarol, phenprocoumon excretion is less influenced by genetic variation of CYP2C9, because phenprocoumon is eliminated both as parent compound (approximately 40%) and hydroxylated metabolites (approximately 60%).[24] Earlier studies on the association of polymorphisms in *CYP2C9* with phenprocoumon maintenance dosage showed no effect [15] or moderate effects only[16, 17].

We further found an association of a *CYP4F2* polymorphism and phenprocoumon maintenance dosage with a weekly dosage increase of 1.5 mg per variant allele with a p-value for significance of 0.023. As we tested 5 candidate genes, under correction for multiple testing this proceeds the adjusted p-value of 0.01 for the additive model. However, this may be due to low numbers and the effect of homozygous *CYP4F2* variant alleles still remained significant (p=0.007). Its ability to account for interindividual dosage variation was modest with an additional 1% within the complete model which included the other genetic variants. This is similar to the additional influence of this gene on warfarin and acenocoumarol dosage.[12, 14] Until now the physiological role of CYP4F2 in coumarin response or vitamin K pathway is only partially elucidated. CYP4F2 hydroxylates the tocopherol phytyl side chain as the first step in the inactivation pathway of vitamin E.[27] As this side chain is quite similar to the side chains of the vitamins within the vitamin K group, CYP4F2 catalyzes metabolisation of vitamin K1.[43] Thus within the vitamin K cycle less substrate would be available for the activation of vitamin K-dependent coagulation factors. Variant alleles of rs2108622 change the protein coding sequence and thus decrease CYP4F2 activity [44]. As polymorhpisms were not shown to change the mRNA expression leves of CYP4F2, the loss of enzyme activity may be caused by either affecting the translation or degradation of CYP4F2.[44] Therefore variant alleles of *CYP4F2* are likely to prevent the inactivation of vitamin K, eplaining the need for the demonstrated higher weekly phenprocoumon dosage with each additional *CYP4F2* variant allele of rs2108622.

We did not find an association between phenprocoumon maintenance dosage and rs2740574, a SNP coding for the *CYP3A4*1*B. Associations between this gene and phenprocoumon have been shown so far only in vitro.[24] A review concluded that CYP3A activity is affected by multiple non-genetic factors and that to date no single genetic defect is known to determine the total metabolic clearance of CYP3A4 substrates.[45] For the *ApoE* genotypes we found a very small clinically neglectable trend of a marginal phenprocoumon dosage decrease of 0.005 mg/week with each additional ε3 and ε4 allele. Possibly due to low numbers, we could not detect associations with a specific allele. In 1,490 acenocoumarol treated patients from the Rotterdam Study we have earlier shown that those with homozygous ε4 allele required a lower maintenance dosage (-1 mg acenocoumarol /week, p=0.02) and those being homozygous for the ε2 allele needed dosage increase (+3,5 mg acenocoumarol /week, p=0.04).[28]

With the polymorphisms of the three genes found to influence phenprocoumon dosage, we built a model, explaining 46% of phenprocoumon maintenance dosage variation. To our knowledge, this is the first study on the combined effects of polymorphisms in *VKORC1*, *CYP2C9* and *CYP4F2* genes on phenprocoumon maintenance dosage. We consider the chance of bias and confounding in this study as negligible. The Rotterdam study is a prospective cohort study and the regional anticoagulation clinic covers a complete area of more than one million inhabitants in the Rotterdam region. Consequently, everyone who is treated with a coumarin anticoagulant as an outpatient will be registered as such and selection bias is highly unlikely. Because data were gathered prospectively, also information bias is unlikely. Phenprocoumon dosage is a well defined phenotype and our results are biologically plausible.

The associations of *VKORC1* and *CYP2C9* with maintenance dosages of all three coumarins represent one of the most promising applications of pharmacogenetics to date. In Caucasians, the benefit from genetic forecasting on warfarin dosage has already been shown [46] and a multicentre study in seven European countries for all three coumarins is underway [47]. If that study can show that genotype-guided dosing can improve the percentage of follow-up time witihin therapeutic range, these findings can be used to increase safety of patients treated with coumarins. Genotyping prior to therapy might help to decide whether a subject with variant alleles of *VKORC1* and *CYP2C9* should preferably be treated with a thrombin inhibitor such as dabigatran instead of treatment with a coumarin.

In conclusion, we demonstrated that apart from therapeutic considerations such as indication and target INR, phenprocoumon maintenance dosage mainly depends on polymorphisms in the *VKORC1* gene. Although of modest effect size, *CYP2C9* and *CYP4F2*, are also relevant for phenprocoumon dosage.

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Chapter 4

Drug interactions with acenocoumarol

Chapter 4.1

Proton pump inhibitors and the risk of overanticoagulation during acenocoumarol maintenance treatment

Abstract

In the Netherlands, several reports described a potentiation of acenocoumarol-induced anticoagulation by co-medication of (es)omeprazole. Our objective was to investigate the effects of co-medication with PPIs on overanticoagulation during acenocoumarol maintenance treatment. All subjects from the Rotterdam Study who received acenocoumarol maintenance treatment between April 1st, 1991 and September 9th, 2009 were followed for events of an international normalized ratio (INR) ≥6, until death, end of treatment, or end of the study period. With the Andersen-Gill extension of the Cox proportional hazards model, risks for repeated events of overanticoagulation in relation to concomitant PPI use were calculated. The risk for overanticoagulation was most pronounced for esomeprazole (HR 1.99, 95% CI 1.55 – 2.55) and lansoprazole (HR 1.49, 95% CI 1.05 – 2.10). There was also a lower and non-significant risk increase for the other PPIs. In conclusion, caution should be paid to co-medication with esomeprazole and lansoprazole (and possibly also with other PPIs) during acenocoumarol treatment.

Introduction

Coumarin anticoagulants are first choice in treatment and prevention of arterial or venous thrombosis.[1] Warfarin, acenocoumarol and phenprocoumon act as vitamin-K antagonists by inhibiting the synthesis of coagulation factor II, VII, IX and X. Due to a particularly narrow therapeutic range, patients treated with these drugs have to be closely monitored by regular assessments of the international normalized ratio (INR) to warrant anticoagulation without serious bleedings. Individual dosing schemes differ widely between patients, mainly due to genetic variation in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 2C9 (*CYP2C9*) genes. In addition, age, sex and body mass index (BMI) are important determining factors.[2-9] Therefore, coumarin dosage is carefully titrated during an initiation period by reference to the INR to achieve a stable individual maintenance dosage. However, also within the maintenance period, effects of coumarins can vary due to interactions with co-medication, development of co-morbidity or changes in lifestyle. In the Netherlands, acenocoumarol and phenprocoumon are most frequently used whereas warfarin is only given on rare occasions. Although management of coumarin use in the Netherlands is constantly monitored in anticoagulation clinics by regular INR-measurements to obtain optimal INR-levels, bleeding associated with coumarins is among the leading causes of drug-induced hospital admissions.[10, 11] Over the years, different drugs were reported to increase the anticoagulation effect of coumarins.[12, 13] Until April 2009, the Netherlands' national pharmacovigilance center (LAREB) received nine reports about an increase of the anticoagulant effect of acenocoumarol within eleven days after start of treatment with omeprazole and seven reports in combination with esomeprazole.[14] Cases of coumarin potentiation by other PPIs have not been reported. In four of these reports, the INR rose above six. INR measurements rising above four are increasingly associated with overanticoagulation, and above six the chance of serious bleedings strongly increases.[15] Competitive inhibition of CYP2C19 has been suggested as a possible mechanism for interactions of PPIs with other drugs.[13] Although earlier studies did not show changes in acenocoumarol pharmacokinetics or pharmacodynamics[16] or a need of acenocoumarol dosage adjustment [17], the Dutch Federation of Anticoagulation Clinics added esomeprazole and omeprazole to the list of drugs potentially interacting with coumarins as of January 2010.[18] Before that date, Dutch computerized medication surveillance systems of GPs and pharmacists did not flag interactions between acenocoumarol and PPIs.

In a large prospective population-based cohort study, we investigated whether co-medication with the PPIs omeprazole, pantoprazole, lansoprazole, rabeprazole or esomeprazole was associated with an increased risk of overanticoagulation during acenocoumarol maintenance treatment and whether an effect was modified by *CYP2C19* variant alleles.

Methods

Setting

We selected all subjects from the three cohorts of the Rotterdam Study (RS-I, RS-II and RS-III). The rationale and design of the Rotterdam Study have been described elsewhere.[19-21] In brief, the Rotterdam Study is a prospective population-based cohort study, designed to study neurological, cardiovascular, locomotor and ophthalmologic diseases in a population of people of 45 years and older. During different periods, eligible inhabitants of Ommoord, a suburb of Rotterdam, were invited to participate. The RS-I cohort consisted of 7,983 subjects (response rate 78%), the RS-II cohort of 3,011 participants (response rate 67%) and the RS-III cohort of 3,932 subjects (response rate 65%). The RS-I cohort had baseline examinations during 1990–1993 with completion of standardized questionnaires, blood sampling and DNA isolation. The RS-II was formed as an independent cohort in 1999 with baseline examinations between 2000 and 2001 and the RS-III cohort was examined between 2006 and 2008.

A regional anticoagulation clinic, Star Medical Diagnostic Center, monitors all inhabitants of Ommoord with an indication for anticoagulant therapy. This clinic covers the patients from all three RS cohorts. From this clinic, since 1984, all data on dosing, laboratory and clinical data, including data on bleeding complications are fully computerized. The patients own treating physician decides about the type of anticoagulant. Prothrombin times are monitored every 1-6 weeks, depending on the target level and stability of the INR. Coumarin doses are adjusted on the basis of computerized dose calculations. More than 99% of participants fill their drug prescriptions at seven regional pharmacies, which are fully computerised. Complete data on drug use from these pharmacies were available as of January^{1st}, 1991. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code[22], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs. As PPIs we included omeprazole (selected from pharmacy dispensing data by the ATC code of the WHO [22], A02BC01), pantoprazole (A02BC02), lansoprazole (A02BC03), rabeprazole (A02BC04) and esomeprazole (A02BC05).

Cohort and outcome definition

Our study population consisted of all patients of the three RS cohorts who started with acenocoumarol in the study period from April 1st, 1991 through September 9th, 2009 and used it consecutively for at least 42 days. The start date of April 1st was chosen to ensure that at least 3 months of medication history from the pharmacy was available for each cohort member. We regarded the period starting 42 days after initiation with acenocoumarol as maintenance period. In general, steady state of a drug is usually achieved within 5-7 half-lives of drug elimination. For (R-)acenocoumarol, the enantiomeric form on which treatment effects mainly depend, with a half-life of 8 hours, a period of 6 weeks taken as initiation period was considered extensive enough to reach a steady state.[23] In patients from the Rotterdam

study using acenocoumarol during the maintenance period, we took an event of an INR of 6 and greater after baseline study enrolment as an outcome. INR levels ≥6 are associated with an extensively increased risk of bleedings.[15] We excluded INR events of six and greater that happened within 21 days of an earlier event.

Cofactors

The following baseline patient characteristics were considered as potential confounders or effect modifiers: sex, age, BMI, and target INR level. BMI was defined as (kg/m^2) and missing values were imputed with a linear regression model consisting of INR≥6, age, sex and target INR as variables. We further adjusted for oral non-steroidal anti-inflammatory drugs (NSAIDs) as co-medication (ATC code M01A), since PPIs might have been started because of treatment with an NSAID and NSAIDs have been shown earlier to increase acenocoumarol effectiveness.[24]

In a subanalysis we studied the association of acenocoumarol maintenance treatment with use of ranitidine (A02BA02). This drug is used for the same indication and not known to be dependent on biotransformation via CYP2C19. [25] In further subanalyses, we studied effect modification of the *CYP2C19*2* variant allele (rs4244285) on an INR ≥6 during use of acenocoumarol. To date, about 19 variant alleles of CYP2C19 have been identified.[26] The majority of individuals can be classified into three phenotype groups, homozygous and heterozygous extensive metabolizers and poor metabolizers. They are determined by the wild-type genotype and two mutated alleles, *CYP2C19*2* and *CYP2C19*3*. The principal defect, *CYP2C19*2* (rs4244285) with a G-allele for normal function and an A-allele as variant with decreased efficiency, leads to a truncated protein and was genotyped in our population. *CYP2C19*3* is rare in Caucasians and was not genotyped in the Rotterdam Study.[15]

Genotyping

All RS participants with available DNA were genotyped using Illumina Infinium II HumanHap BeadChips at the Department of Internal Medicine, Erasmus Medical Center following manufacturer's protocols. RS-I participants (n=6,449) were genotyped with 550k (V.3) single and duo chips, while RS-II participants (n=2,516) were genotyped with 550k (V.3) duo and 610k Quad chips. RS-III (n=2.420) participants were genotyped with the Human 610 Quad Arrays of Illumina. Genotype calling was performed in RS-I using BeadStudio software (version 0.3.10.14), GenomesStudio in RS-II and Bead Studio (v3.2.23) in RSIII. Participants with call rates <97.5%, excess autosomal heterozygosity, sex mismatch or outlying identity-by-state clustering estimates were excluded. After quality control, 5,974 RS-I participants, 2,157 RS-II and 2,078 RS-III participants remained with complete data on genotyping.[27] For imputation, 512,349 autosomal SNPs in RS-I and 466,389 autosomal SNPs in RS-II and RS-III were used after exclusions for call rate < 98%, HWE $P<10^{-6}$, and MAF <1%, in MACH (version 1.00.15 for RS-I, 1.00.16 for RS-II and RS-III) with reference to the 2,543,886 SNPs of the HapMap CEU (release 22, build 36).[27]

Statistical analysis

Within a subject, an INR ≥6 could occur more than once and co-medication with PPIs could change during acenocoumarol treatment period. In order to include all information available for the whole study period, we used the Andersen-Gill extension of the Cox proportional hazards model. This model allows to study multiple events of an INR ≥6 within one subject and in association with PPI use as a time-varying covariable.[28] All subjects on acenocoumarol maintenance therapy were followed as of April 1st, 1991, from their first INR assessment until the last INR assessment because of the end of their treatment, last INR ≥6 event or the end of the study period, whichever came first. The date on which an INR ≥6 occurred was taken as the index date. In order to exclude protopathic bias, patients were only considered as exposed to PPIs at the index date if they had started PPI at least 3 days before that date. Each case was compared for PPI exposure to all subjects who were on acenocoumarol maintenance treatment at the index date.[29] Thus cases could serve as controls on other index dates when still being on acenocoumarol treatment. We computed hazard ratios (HRs) and their 95% confidence intervals (CIs) for all events of an INR≥6. Risk estimates were adjusted for age, sex, target INR, BMI and NSAID co-medication. To study effect modification by *CYP2C19* genotype, patients were stratified according to their genotype as *CYP2C19*2* homozygous G-alleles and variant type (*CYP2C19*2* heterozygous G/A-alleles or *CYP2C19*2* homozygous A-alleles).

SPSS 15.0 was used for data management and SAS 9.20 for the Andersen-Gill analysis.

Results

Of the 14,926 subjects in the Rotterdam cohorts, 2,755 had used acenocoumarol during the study period for longer than 42 days continuously as maintenance therapy. In 887 subjects an INR ≥6 was measured at least once and in total 2146 INR ≥6 occurred, between 1 and 22 events per subject at a median of 2 per person. Baseline characteristics of patients with an INR ≥6 and the total cohort are shown in table 1. 43% subjects with acenocoumarol maintenance therapy were male, mean age at study entry was 69 years and mean BMI 27.2 kg/m^2. BMI values had to be imputed in 280 subjects (10.9%). Increasing age and higher INR target levels significantly increased the risk to develop an INR≥6. Higher BMI measures were associated with a decreased risk of overanticoagulation. From the 2,059 subjects successfully genotyped for *CYP2C19*2*, 1,464 (71.1%) were homozygous for the *CYP2C19*2 G-allele* and 545 (28.9%) had a variant genotype with an A-allele. The frequency of the *CYP2C19*2* variant allele A was 15.7% and alleles were in Hardy-Weinberg equilibrium (HWE, p-value=0.93). Subjects homozygous with the variant A-allele of *CYP2C19*2* had a decreased risk on overanticoagulation during acenocoumarol treatment (HR 0.54; 95% CI 0.36-0.83). As this group was quite small (2.4%), we pooled subjects homozygous and heterozygous for the variant A-allele. Any variant A-allele was then no longer associated with the risk of overanticoagulation (HR 0.99; 95% CI 0.89-1.11).

Table 1 Characteristics of acenocoumarol users with INR of 6.0 or greater and total cohort

	Patients with INR>=6.0 (N=887)	Total cohort (N=2,755)	HR[a]	95% CI
Gender				
Male (%)	405 (45.7)	1,192 (43.3)	1.00	Reference
Female (%)	482 (54.3)	1,563 (56.7)	**1.25**	**(1.15 – 1.36)**
Start age (years)				
45 – 54	10(1.1%)	114 (4.2%)	1.00	Reference
55 – 64	72 (8.1%)	383 (14.0%)	**2.89**	**1.75 – 4.78**
65 – 74	214 (24.1%)	907 (33.2%)	**2.74**	**1.69 – 4.45**
75 – 84	389 (43.9%)	998 (36.5%)	**4.14**	**2.56 – 6.68**
>85	202 (22.8%)	334 (12.2%)	**6.10**	**2.63 – 14.1**
BMI (kg/m^2)				
15.0 – 20.0	17 (1.9)	45 (1.6)	1.00	Reference
20.1 – 25.0	228 (25.7)	718 (26.1)	**0.70**	**0.52 – 0.93**
25.1 – 30.0	492 (55.5)	1446 (52.6)	**0.68**	**0.51 – 0.91**
>30.0	150 (16.9)	539 (19.6)	**0.56**	**0.42 – 0.76**
Target level (INR)				
2.0 – 2.5	1 (0.1)	118 (4.3)	1.00	Reference
2.0 - 3.5	389 (43.9%)	1728 (62.7%)	**10.1**	**3.23 – 31.2**
3.0 - 4.0	482 (54.3%)	889 (32.3%)	**27.9**	**9.00 – 86.7**
3.5 - 4.5	15 (1.7%)	20 (0.7%)	**65.4**	**17.3 - 247**
Subjects with NSAID use	89 (10.0)	470 (17.0)	**1.31**	**1.15 – 1.50**
*CYP2C19*2* genotypes[b]	N=648 (73.1% of all cases)	N=2059 (74.7% of the total group)		
GG	472 (72.8%)	1464 (71.1%)	1.00	Reference
GA	167 (25.8%)	545 (26.5)%	0.96	0.87 – 1.07
AA	9 (1.4%)	50 (2.4%)	**0.54**	**0.36 – 0.83**

Statistically significant values are printed in bold

a Univariate analysis of RR were performed with an Andersen-Gill model. RRs cannot be calculated with the numbers in this table because controls may later become cases; significant values are printed in bold.

b For the CY2C19*2 genotype within total cohort, allelic frequency of the G-allele was 84.3%, of the A-allele 15.7%, and HWE was 0.93

Abbreviations: INR, international normalized ratio; HR, hazard ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

In our cohort in total 457 (16.6%) subjects used omeprazole, 160 (5.8%) pantoprazole, 102 (3.7%) lansoprazole, 286 (10.4%) rabeprazole and 149 (5.4%) esomeprazole (table 2). In 223 events of an INR ≥6 a PPI (10.4% of all events) was used on the index date for at least three days. Co-medication during acenocoumarol maintenance treatment with esomeprazole was associated with a doubled risk of an INR ≥6 (HR 1.99; 95% CI 1.55–2.55) and co-medication with lansoprazole increased the risk by 49 percent (HR 1.49; 95% CI 1.05–2.10, table 2). The other PPIs also increased the risk of overanticoagulation less pronounced between 1.12 and 1.23 times, but associations were just below the significance threshold after adjustment for co-medication with NSAIDs. Co-medication with ranitidine, used by 229 subjects (10.5%), showed no association with overanticoagulation (HR 1.06, 95% CI 0.83–1.35).

For esomeprazole and lansoprazole we investigated whether the CYP2C19 genotype modified the interaction with acenocoumarol (table 3). We did not find a multiplicative in-

Table 2 Association between overanticoagulation (INR≥6) under acenocoumarol maintenance treatment and proton pump inhibitors

PPI	*Total cohorta (N=2,755) and use of a specific drug*	**Andersen-Gill analysis[a]**		
		Cases with use of a specific drug with at least one INR≥6.0	HR[b] (95% CI)	HR[c] (95% CI)
Omeprazole	457	82	**1.18 (1.01 – 1.38)**	1.12 (0.96 – 1.32)
Pantoprazole	160	31	1.27 (0.97 – 1.67)	1.21 (0.92 – 1.59)
Lansoprazole	102	24	**1.50 (1.06 – 2.12)**	**1.49 (1.05 – 2.10)**
Rabeprazole	286	44	**1.30 (1.01 – 1.67)**	1.23 (0.95 – 1.58)
Esomeprazole	149	42	**2.08 (1.63 – 2.67)**	**1.99 (1.55 – 2.55)**
Subanalysis				
Ranitidine	290	44	1.08 (0.84 – 1.35)	1.06 (0.83 – 1.35)

Statistically significant values are printed in bold

a In this time-dependent analysis, exposure in case patient and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). As 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 HRs cannot be calculated from the data in this table.

b adjusted for age, sex, BMI and target INR

c adjusted for age, sex, BMI, target INR and use of non-steroidal anti-inflammatory drugs, ATC-group M01A.

Table 3 Association between overanticoagulation (INR≥6.0), esomeprazole and lansoprazole, stratified by ***CYP2C19****2 genotype

	HR[a] (95% CI)	P-value of multiplicative interaction
Interaction between esomeprazole and *CYP2C19*2* genotypes (homozygous G-allele / at least one variant A-allele)		0.15
No use of esomeprazole, *CYP2C19*2* homozygous G-allele	1.00 reference	
No use of esomeprazole, *CYP2C19*2* at least one variant A allele	0.94 (0.84 – 1.04)	
Use of esomeprazole, *CYP2C19*2* homozygous G-allele	**1.97 (1.47 – 2.64)**	
Use of esomeprazole, *CYP2C19*2* at least one variant A allele	1.05 (0.47 – 2.34)	
Interaction between esomeprazole or lansoprazole and *CYP2C19*2* genotypes (wild type genotype / variant alleles)		0.73
No use of esomeprazole or lansoprazole, *CYP2C19*2* homozygous G-allele	1.00 reference	
No use of esomeprazole or lansoprazole, *CYP2C19*2* at least one variant A allele	0.92 (0.83 – 1.03)	
Use of esomeprazole or lansoprazole, *CYP2C19*2* homozygous G-allele	**1.69 (1.31 – 2.19)**	
Use of esomeprazole or lansoprazole, *CYP2C19*2* at least one variant A allele	1.52 (0.93 – 2.50)	

Statistically significant values are printed in bold

a adjusted for age, sex, BMI, target INR and use of nonsteroidal anti-inflammatory drugs, ATC-group M01A.

teraction between the drugs separately or grouped for use of either of the drugs and at least one *CYP2C19*2* variant allele. To investigate presence of an additive effect modification we formed 4 groups for the combinations of drug use yes and no with absence and presence of *CYP2C19*2* variant alleles. No additive effect modification was detected either.

Discussion

In our study population, all PPIs tended to increase the risk of overanticoagulation during acenocoumarol maintenance treatment. This was most pronounced for esomeprazole, doubling the risk of overanticoagulation and for lansoprazole with a risk increase of 49%. Risk increases for omeprazole, pantoprazole and rabeprazole were between 12 and 23% and just failed to reach significance after adjustment for NSAID co-medication.

PPIs have been mentioned occasionally as potential risk factors for overanticoagulation during treatment with vitamin K antagonist in case reports, however most case control studies and trials concluded no effect of clinical relevance.[12, 17] The highest number of case reports and pharmacokinetic studies are available for omeprazole in combination with coumarins. Two case reports described an INR increase in users of phenprocoumon after initiation of omeprazole and return to normal after its cessation.[30] One case controls study found no evidence for an interaction between omeprazole and acenocoumarol. They used acenocoumarol dose adjustment as an outcome which did not differ between a group of acenocoumarol users with omeprazole as co-medication and a group acenocoumarol users without interfering co-medication.[16] However, in contrast to dosage adjustments, INR measures are directly related to acenocoumarol effects. A pharmacokinetic study showed that omeprazole increased plasma concentration of R-warfarin by nearly 10% whereas there was no effect on S-warfarin, the more active isomer.[31] The authors concluded that an effect of omeprazole on the anticoagulation activity of warfarin was not likely to be of clinical importance. This finding was confirmed by a randomized double-blind cross-over-study where coagulation time of warfarin during omeprazole use was not significantly changed compared to placebo.[32] Also within acenocoumarol users, one cohort study and one trial found no evidence for an interaction between acenocoumarol and omeprazole.[16, 17] For pantoprazole, a trial showed no effect on phenprocoumon treatment. [33] However, the trials were only short term trials with small numbers of subjects and the cohort study took dosage change as an outcome without regard to INR measurements. In one study for co-medication with lansoprazole or rabeprazole in patients after open heart surgery,[34] lansoprazole enhanced the anticoagulation effects of warfarin whereas rabeprazole could be used concomitantly without increasing the risk of overanticoagulation. We are not aware of a clinical study on the effect of esomeprazole on coumarins to support our findings.

As a possible mechanism a competitive inhibition of CYP2C19 by PPIs on coumarin clearance has been mentioned as both drug groups are metabolized by this enzyme and the INR increases in the case reports were reported within 11 days after start of the PPI.[13, 35, 36] Within coumarins, the (R-)enantiomeric form of warfarin and both forms of acenocoumarol are metabolized by CYP2C19.[13, 37] In general, the (S-)isomers of the coumarins are more effective. However, for acenocoumarol, the (S-)form has an extremely short half live of 2 hours and acenocoumarol effects mainly depend on the (R-)enantiomeric form of which about 20%

is metabolized via CYP2C19. Therefore, for acenocoumarol more than for warfarin, a pharmacokinetic interaction with PPIs via CYP2C19 might lead to an effect of clinical relevance. Clearance of PPIs is conducted primarily by CYP2C19. In vitro studies showed that all five PPIs were able to competitively inhibit CYP2C19, but to a different extent. Lansoprazole had the highest inhibitory potency of CYP2C19 in vitro.[38] Rabeprazole had a relatively lower in vitro inhibition of CYP2C19 than the other PPIs, possibly as it is metabolized mainly via a non-enzymatic reaction to a thioether compound with only minor CYP2C19 involvement. [34, 36, 38] Omeprazole and esomeprazole in vitro also reduced CYP2C19 activity [38] and were more potent than pantoprazole.[39] Theoretically a competition for scarce CYP2C19 enzyme might prolong the effectiveness of the coumarins which would result in an increased INR. In subjects with variant allele genotypes and decreased enzyme availability this effect may be even more pronounced. However, from our analysis, no multiplicative or additive effect modification of *CYP2C19**2 variant alleles on the association between esomeprazole or lansoprazole and acenocoumarol was detected. Possibly the effect of the variant allele on acenocoumarol elimination was too small and more cases are needed to warrant enough power for a genotype-stratified analysis.

Our results show a more pronounced association for esomeprazole than for omeprazole. Omeprazole is a racemic composition of its two optical isomers, (S-)omeprazole (esomeprazole) and (R-)omeprazole.[40] The clearance of the two enantiomeric omeprazole-forms by CYP2C19 is stereo-selective and depend for (R-)omeprazole more on the CYP2C19 enzyme than for (S-)enantiomeric form.[40-42] In vivo, CYP2C19 was responsible for 90% of the metabolism of (R-)omeprazole and 70% of (S-)omeprazole.[42] The interaction potential for esomeprazole, however, was expected to be similar to omeprazole because of the higher standard dose used for esomeprazole (40 mg) compared to omeprazole (20 mg) in clinical practice and lower clearance of esomeprazole. [25, 38, 42] In our study population, omeprazole and esomeprazole were used in similar defined daily doses (DDD), omeprazole at a mean DDD of 1.35 (range 0.21–4.50) and esomeprazole at a mean DDD of 1.23 (range 0.33–4.00). However, these DDDs do not represent equipotent dosages as 1 DDD omeprazole equals 20 mg and 1 DDD esomeprazole 30 mg. Consequently in our study population, esomeprazole was used in a much higher pharmacological dosage than omeprazole.

Our study is the first observational cohort study of an increase of acenocoumarol effectiveness by co-medication of all PPIs separately with a substantial number of subjects during the whole period of acenocoumarol maintenance treatment within a subject. We consider the chance of bias and confounding due to study design as negligible. First, the Rotterdam Study is a prospective cohort study and the regional anticoagulation clinic covered a complete area of more than one million inhabitants in the Rotterdam area. Consequently, everyone who is treated with a coumarin anticoagulant as an outpatient will be registered as such and selection bias is unlikely. Second, all medication use of all subjects was almost completely covered by the pharmacy data we retrieved and during the study period PPIs were only avail-

able on prescription via pharmacies. Any lack of compliance would move our results into the direction of the null hypothesis, and would tend to make our results conservative. During our study period, an association between PPI and acenocoumarol overanticoagulation was not yet common knowledge. Third we followed the cohort members during their whole period of acenocoumarol maintenance therapy and compared cases to all other cohort members available at the index dates for PPI exposure. The analysis made use of all data available and adjusted for time varying effects. However, in observational studies there is always a risk of confounding by indication. We therefore performed the same analysis for rantidine, a H2-receptor antagonist used for the same indication but not known for inhibitory potential of CYP2C19. We did not find an association for ranitidine with an increased risk on achieving INR ≥6 during acenocoumarol treatment. As PPIs might have been started to treat the symptoms of an increased INR, in our analysis use of PPIs – and also ranitidine – had to be started for at least 3 days prior to the index date. We did not take bleedings as an outcome because PPI s are likely to be started with complaints preceding gastrointestinal bleeding. Associations found might be due to protopathic bias.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic using acenocoumarol for maintenance treatment, esomeprazole doubled the risk on an INR ≥6 and lansoprazole tended to increase this risk by approximately 50%. For omeprazole, pantoprazole and rabeprazole, a risk increase was less pronounced and non-significant. Our results suggest that extra monitoring during acenocoumarol treatment may be warranted in patients on esomeprazole or lansoprazole and this perhaps applies to other PPIs as well.

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Chapter 4.2

Selective serotonin reuptake inhibitors and the risk of overanticoagulation during acenocoumarol maintenance treatment

Abstract

Potentiation of warfarin-induced anticoagulation by co-medication with selective serotonin reuptake inhibitors (SSRIs) has been described previously. Our objective was to investigate the effects of co-medication with SSRIs on overanticoagulation during acenocoumarol maintenance treatment. All subjects from the Rotterdam Study who received acenocoumarol maintenance treatment between April 1st, 1991 and September 9th, 2009 were followed for the event of an international normalized ratio (INR) ≥6, until death, end of treatment or end of the study period. With the Andersen-Gill extension of the Cox proportional hazards model, risks for repeated events of overanticoagulation in relation to concomitant SSRI use were calculated. The risk for overanticoagulation during acenocoumarol maintenance treatment was increased in combination with fluvoxamine (HR 2.63, 95% CI 1.49–4.66) and venlafaxine (HR 2.19, 95% CI 1.21–3.99) but not for the other SSRIs. In conclusion, fluvoxamine and venlafaxine were associated with a more than double risk of INR measures ≥6 in acenocoumarol treated subjects.

Introduction

Coumarin anticoagulants are first choice in treatment and prevention of arterial or venous thrombosis.[1] Warfarin, acenocoumarol and phenprocoumon act as vitamin-K antagonists by inhibiting the synthesis of coagulation factor II, VII, IX and X. Due to a particularly narrow therapeutic range, patients treated with these drugs have to be closely monitored by regular assessments of the international normalized ratio (INR) to warrant anticoagulation without serious bleedings. Individual dosage differs widely between patients, mainly due to genetic variation in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450 2C9 (*CYP2C9*) genes in combination with age, sex and body mass index (BMI).[2-9] Therefore during an initiation period, coumarin dosage is carefully titrated by reference to the INR to achieve a stable individual maintenance dosage. However, also within the maintenance period effects of coumarins can vary due to interactions with co-medication or development of co-morbidity. In the Netherlands, acenocoumarol and phenprocoumon are most frequently used whereas warfarin is only given on rare occasions. Although management of coumarin use in the Netherlands is constantly monitored in anticoagulation clinics by regular INR-measurements to obtain optimal INR-levels, bleeding associated with coumarins is among the leading causes of drug-induced hospital admissions.[10, 11]

Selective serotonin reuptake inhibitors (SSRIs) have also been reported to increase the risk of bleeding [12-17]. Increase of prothrombin time was described in several case reports for fluvoxamine and fluoxetine in combination with warfarin. [18-21] Results from a case control study for the combination of acenocoumarol or phenprocoumon with SSRIs showed a risk increase for non gastrointestinal bleeding, but not for gastrointestinal bleeding.[22] Another case control study reported a small risk increase for upper gastrointestinal haemorrhage with SSRIs which was most pronounced for venlafaxine.[23] However, there was no evidence for effect modification on bleeding risk for SSRI use in combination with warfarin. [23] A further case control study on initiation of SSRIs in warfarin users found no significant risk increase of hospitalization for upper gastrointestinal bleeding.[24] The Dutch Federation of Anticoagulation Clinics included the group of SSRIs in the list of drugs for extra monitoring during coumarin treatment although the separate contribution of individual SSRIs and the exact underlying mechanism of the interaction are not yet known.[25] The mechanism might be pharmacodynamic as serotonin release is important for the platelet aggregation and inhibition of its reuptake by SSRIs might prevent haemostasis additional to the effects on anticoagulation by coumarins.[14] The mechanism of the interaction might also be pharmacokinetic as fluvoxamine and sertraline were reported to inhibit CYP2C9, the main metabolizing enzyme of coumarins.[26] This would increase coumarin effectiveness and might result in increase of INR and bleedings. In a large prospective population-based cohort study, we investigated whether co-medication with the SSRIs fluoxetine, citalopram, paroxetine, sertraline, fluvoxamine, escitalopram or venlafaxine was associated with an increased risk of

overanticoagulation during acenocoumarol maintenance treatment and whether an effect was modified by *CYP2C9* variant alleles.

Methods

Setting

We selected all subjects from the three cohorts of the Rotterdam Study (RS-I, RS-II and RS-III). The rationale and design of the Rotterdam Study have been described elsewhere.[27-29] In brief, the Rotterdam Study is a prospective population-based cohort study, designed to study neurological, cardiovascular, locomotor and ophthalmologic diseases in a population of people of 45 years and older. The RS-I cohort consisted of 7,983 subjects (response rate 78%), the RS-II cohort of 3,011 participants (response rate 67%) and the RS-III cohort of 3,932 subjects (response rate 65%). The RS-I cohort had baseline examinations during 1990–1993 with completion of standardized questionnaires, sampling of blood and isolation of DNA. The RS-II was formed as an independent cohort in 1999 with baseline examinations between 2000 and 2001 and the RS-III cohort was examined between 2006 and 2008.

A regional anticoagulation clinic, Star Medical Diagnostic Center, monitors all inhabitants of Ommoord, a suburb of Rotterdam, with an indication for anticoagulant therapy. This clinic covers the patients from all the RS cohorts. From this clinic, since 1984, all data on dosing, laboratory and clinical data, including data on bleeding complications are fully computerized. The physician who treats the patient decides about the type of anticoagulant. Prothrombin times are monitored every 1-6 weeks, dependent on the target level and stability of the INR. Coumarin doses are adjusted on the basis of computerized dose calculations. More than 99% of participants fill their drug prescriptions at seven regional pharmacies, which are fully computerised. Complete data on drug use from these pharmacies were available as of 1 January 1991. In order to assess initiation of SSRIs, we used data from 1st April, 1991 through 9th September, 2009. The pharmacy data include the Anatomical Therapeutical Chemical (ATC)-code[30], the filling date, the total amount of drug units per prescription, the prescribed daily number of units, and product name of the drugs. As SSRIs we included fluoxetine (ATC code N06AB03), citalopram (N06AB04), paroxetine (N06AB05), sertraline (N06AB06), fluvoxamine (N06AB08), escitalopram (N06AB10) and venlafaxine (N06AX16).

Cohort and outcome definition

Our study population consisted of all patients of the three RS cohorts who started with acenocoumarol in the study period between 1st April 1991 and 9th September 2009, using it consecutively for at least 42 days. We regarded a treatment period starting 42 days after initiation with acenocoumarol as maintenance period. Steady state of a drug is usually achieved within 5-7 half-lives of drug elimination. The (S-)enantiomeric form of acenocoumarol has a

2-5 fold higher anticoagulant potency than the (R-)form, however due to the extremely fast clearance of the (S-)enantiomeric, treatment effects are mainly due to (R-) acenocoumarol. For (R-)acenocoumarol with a half-life of 8 hours, a period of 6 weeks taken as initiation period was considered extensive enough to reach a steady state.[31] In patients from the Rotterdam study using acenocoumarol during the maintenance period, we took an event of an INR of 6 and greater after baseline study enrolment as an outcome. INR levels ≥ 6 are associated with an extensively increased risk of bleedings.[32] An assessment of the INR ≥ 6 occurring in a particular individual within 21 days of an earlier event was considered as part of one event episode. When occurring more than 21 days after an earlier event, it was considered as a new event.

Cofactors

The following baseline patient characteristics were considered as potential confounders or effect modifiers: sex, age, BMI, and target INR level. BMI was defined as (kg/m^2) and missing values were imputed with a linear regression model consisting of INR≥6, age, sex and target INR as variables. We further adjusted for co-medication with oral non-steroidal anti-inflammatory drugs (NSAIDs, M01A) and proton pump inhibitors (A02BC) as these drugs have been shown to interact with acenocoumarol with effects on INR measures.[33, 34]

We did not adjust for co-medication with platelet inhibitors (B01AC), acetyl salicylic acid (N02BA), and corticosteroids (H02AB, H02BX), as these drugs do not influence the INR assessment. We verified the independency of our results from this co-medication in an additional analysis (results not given). In a sub-analysis we studied the interaction between acenocoumarol maintenance treatment with the use of nortriptyline (N06AA01) or mirtazapine (N06AX11).These are frequently prescribed antidepressants that are not metabolized via CYP2C9 and thus unlikely to cause a pharmacokinetic interaction. Nortriptyline and mirtazapine furthermore have no significant affinity for the serotonin transporter[35] and consequently are not likely to show a pharmacodynamic interaction either. In an additional sub-analysis we studied effect modification of a *CYP2C9* variant (T-) allele (rs4086116) on an INR ≥6 during use of acenocoumarol. Within the CYP2C cluster, this SNP was found to be most strongly associated with acenocoumarol dosage variation and explained nearly as much of dosage variation as the combined *CYP2C9**2/*3 genotypes[36]. This SNP was genotyped in al three RS-cohorts. We did not expect effect-modification by *VKORC1* variant alleles as this gene is not involved in mechanism or kinetics of the SSRIs. We confirmed the independency of this assumption with results in an additional analysis (results not given).

Genotyping

From all RS participants, those with available DNA were genotyped using Illumina Infinium II HumanHap BeadChips at the Department of Internal Medicine, Erasmus Medical Center following manufacturer's protocols. RS-I participants (n=6,449) were genotyped with 550k

(V.3) single and duo chips, while RS-II participants (n=2,516) were genotyped with 550k (V.3) duo and 610k Quad chips. RS-III (n=2.420) participants were genotyped with the Human 610 Quad Arrays of Illumina. Genotype calling was performed in RS-I using BeadStudio software (version 0.3.10.14), GenomesStudio in RS-II and Bead Studio (v3.2.23) in RSIII. Participants with call rates <97.5%, excess autosomal heterozygosity, sex mismatch or outlying identity-by-state clustering estimates were excluded. After quality control, 5,974 RS-I participant, 2,157 RS-II and 2,078 RS-III participants remained with complete data on genotyping.[37] For imputation 512,349 autosomal SNPs in RS-I and 466,389 autosomal SNPs in RS-II and RS-III were used after exclusions for call rate < 98%, HWE $P<10^{-6}$, and MAF <1%, in MACH (version 1.00.15 for RS-I, 1.00.16 for RS-II and RS-III) with reference to the 2,543,886 SNPs of the HapMap CEU (release 22, build 36).[37]

Statistical analysis

Within a subject, an INR ≥6 could occur more than once and co-medication with SSRIs could change during an acenocoumarol treatment period. In order to include all information available for the whole study period, we used the Andersen-Gill extension of the Cox proportional hazards model. This model allows to study multiple events of an INR ≥6 within one subject and uses exposure to SSRIs as a time-varying covariable.[38] All subjects on acenocoumarol maintenance therapy were followed as of April 1st, 1991, from their first INR assessment until the last INR assessment because of the end of their treatment or last INR ≥6 event, the end of study period, or death, whichever came first. The date on which an INR ≥6 occurred was taken as the index date. Each case was compared for SSRI exposure to all subjects who were on acenocoumarol maintenance treatment at the index date.[39] Thus cases could serve as controls on other index dates when still being on acenocoumarol treatment until their last measure of an INR ≥6. We computed hazard ratios (HRs) and their 95% confidence intervals (CIs) for all events of an INR ≥6. Risk estimates were adjusted for age, sex, target INR, BMI and co-medication with NSAID or proton pump inhibitors. To study effect modification by *CYP2C9* genotype, subjects from the study population who were successfully genotyped were stratified as wild type (*CYP2C9* homozygous C-alleles) and variant type (*CYP2C9* heterozygous C/T-alleles or *CYP2C9* homozygous T-alleles). SPSS 15.0 was used for data management and SAS 9.20 for the Andersen Gill analysis.

Results

Of the 14,926 subjects in the Rotterdam cohorts, 2,755 had used acenocoumarol during the study period for longer than 42 days continuously as maintenance therapy. In 887 subjects an INR ≥6 was measured at least once. Baseline characteristics of patients with an INR ≥6 and the total cohort are shown in table 1. From the subjects on acenocoumarol maintenance

Table 1 Characteristics of acenocoumarol users with INR of 6.0 or greater and total cohort

	Patients with INR>=6.0 (N=887)	Total cohort (N=2,755)	HR[a]	95% CI
Gender				
Male (%)	405 (45.7)	1,192 (43.3)	1.00	Reference
Female (%)	482 (54.3)	1,563 (56.7)	**1.25**	**(1.15 – 1.36)**
Start age (years)				
45 – 54	10(1.1%)	114 (4.2%)	1.00	Reference
55 – 64	72 (8.1%)	383 (14.0%)	**2.89**	**1.75 – 4.78**
65 – 74	214 (24.1%)	907 (33.2%)	**2.74**	**1.69 – 4.45**
75 – 84	389 (43.9%)	998 (36.5%)	**4.14**	**2.56 – 6.68**
>85	202 (22.8%)	334 (12.2%)	**6.10**	**2.63 – 14.1**
BMI (kg/m2)				
15.0 – 20.0	17 (1.9)	45 (1.6)	1.00	Reference
20.1 – 25.0	228 (25.7)	718 (26.1)	**0.70**	**0.52 – 0.93**
25.1 – 30.0	492 (55.5)	1,446 (52.6)	**0.68**	**0.51 – 0.91**
>30.0	150 (16.9)	539 (19.6)	**0.56**	**0.42 – 0.76**
Target level (INR)				
2.0 – 2.5	1 (0.1)	118 (4.3)	1.00	Reference
2.0 - 3.5	389 (43.9%)	1728 (62.7%)	**10.1**	**3.23 – 31.2**
3.0 - 4.0	482 (54.3%)	889 (32.3%)	**27.9**	**9.00 – 86.7**
3.5 - 4.5	15 (1.7%)	20 (0.7%)	**65.4**	**17.3 - 247**
Subjects with NSAID use	89 (10.0)	470 (17.0)	**1.31**	**1.15 – 1.50**
Subjects with PPI use	198 (22.3)	862 (31.3)	**1.46**	**1.31 – 1.64**
CYP2C9 genotypes[b]	N=648 (73.1% of all cases)	N=2,059 (74.7% of the total group)		
CC	428 (66.0%)	1,352 (65.7%)	1.00	Reference
CT	192 (29.6%)	629 (30.5)%	0.95	0.86 – 1.05
TT	28 (4.3%)	78 (3.8%)	**1.38**	**1.12 – 1.70**

Abbreviations: INR, international normalized ratio; HR, hazard ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

a Univariate analysis of HR were performed with the Andersen-Gill model. HRs cannot be calculated with the numbers in this table because controls may later become cases; significant values are printed in bold.

b For the CY2C9 genotype within total cohort, allelic frequency of the C-allele was 80.9%, of the T-allele 19.1%, and HWE was 0.96

therapy 43% were male, mean age at study entry was 69 years and mean BMI 27.2 kg/m^2. BMI values had to be imputed in 280 subjects (10.9%). Increasing age and higher INR target levels significantly increased the risk to develop an INR ≥ 6. Higher BMI measures were associated with a decreased risk of overanticoagulation. From the 2,059 subjects successfully genotyped for *CYP2C9*, 1,352 (65.7%) had a wild type genotype and 712 (34.3%) had a variant genotype. The frequency of the *CYP2C9* variant T-allele was 19.1% and alleles were in Hardy-Weinberg equilibrium (HWE, p-value=0.65). In subjects being homozygous for the *CYP2C9* variant allele the risk for an INR ≥6 was increased 1.38 times (95% CI 1.12–1.70).

In our cohort in total 225 subjects (5%) used SSRIs of whom 18 subjects used fluoxetine, 32 citalopram, 97 paroxetine, 23 sertraline, 26 fluvoxamine, 2 escitalopram and 14 venlafaxine (table 2). In 38 events of an INR ≥6 a SSRI (4.3% of all events) was used on the index date. Co-medication during acenocoumarol maintenance treatment with fluvoxamine and

Table 2 Association between overanticoagulation (INR≥6) under acenocoumarol maintenance treatment and SSRIs

SSRI	Number of patients with drug use within total cohort [a] (N=2,755)	Andersen-Gill analysis[a]		
		Cases with use of a specific drug with at least one INR≥6.0	HR[b] (95% CI)	HR[c] (95% CI)
Fluoxetine	18	1	0.50 (0,07 – 3.55)	0.48 (0.07 – 3.40)
Citalopram	32	3	0.62 (0.20 – 1.94)	0.63 (0.20 – 1.95)
Paroxetine	97	18	1.15 (0.81 – 1.65)	0.99 (0.68 – 1.44)
Sertraline	23	2	1.07 (0.34 – 3.34)	1.02 (0.33 – 3.18)
Fluvoxamine	36	8	**2.46 (1.39 – 4.34)**	**2.63 (1.49 – 4.66)**
Escitalopram	2	0	P=0.95	P=0.95
Venlafaxine	14	6	**2.37 (1.37 – 4.10)**	**2.19 (1.21 – 3.99**)
Sub-analysis				
Nortriptyline	12	2	0.60 (0.25 – 1.44)	0.55 (0.21 – 1.47)
Mirtazapine	45	8	1.01 (0.54 – 1.88)	1.09 (0.58 – 2.04)

Statistically significant values are printed in bold

a In this time-dependent analysis, exposure in case patient and in the rest of the cohort is assessed at the time of the outcome in each case patient (index date). As 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 RRs cannot be calculated from the data in this table.

b adjusted for age, sex, BMI and target INR; if none of the cases was exposed, P values are given instead of HRs.

c adjusted for age, sex, BMI, target INR and use of non-steroidal anti-inflammatory drugs (ATC-group M01A) or proton pump inhibitors (A02BC) if none of the cases was exposed, P values are given instead of HRs.

venlafaxine more than doubled the risk on an INR≥6, for fluvoxamine by 2.63 times (95% CI 1.49–4.66) and for venlafaxine by 2.19 times (95% CI 1.21–3.99). The other SSRIs showed no association with overanticoagulation.

For fluvoxamine and venlafaxine we investigated whether the CYP2C9 genotype modified the interaction with acenocoumarol (table 3). We did not find a multiplicative effect measure modification for an interaction between fluvoxamine or venlafaxine and at least one *CYP2C9* variant allele. To investigate presence of an additive effect measure modification we formed 4 groups for the combinations of drug use 'yes'/'no' with absence and presence of *CYP2C9* variant alleles. No additive effect measure modification was detected either.

Discussion

In our study population, use of fluvoxamine and venlafaxine was associated with a more than double risk of overanticoagulation during acenocoumarol maintenance treatment. Co-medication with the other SSRIs, fluoxetine, citalopram, sertraline or escitalopram, was not associated with an INR≥6. Our findings for fluvoxamine are supported by two case reports for INR increase during warfarin treatment.[18, 21] We did not find a study of the effect of venlafaxine on INR measures. In agreement with our results, clinical drug trials with fluox-

Table 3 Association between overanticoagulation (INR ≥6.0), exposure to fluvoxamine or venlafaxine, stratified by CYP2C9 genotype

	RR[a] (95% CI)	P-value of multiplicative interaction
Interaction between fluvoxamine and CYP2C9 genotype (wild type genotype / variant alleles)		0,48
No use of fluvoxamine, CYP2C9 wild type genotype	1.00 reference	
No use of fluvoxamine, CYP2C9 variant alleles	1.08 (0.98 – 1.18)	
Use of fluvoxamine, CYP2C9 wild type genotype	1.99 (0.64 – 6.20)	
Use of fluvoxamine, CYP2C9 variant alleles	1.22 (0.30 – 4.89)	
Interaction between venlafaxine and CYP2C9 genotype (wild type genotype / variant alleles)		0,56
No use of venlafaxine, CYP2C9 wild type genotype	1.00 reference	
No use of venlafaxine, CYP2C9 variant alleles	1.08 (0.98 – 1.19)	
Use of e venlafaxine, CYP2C9 wild type genotype	**2.13 (1.10 – 4.12)**	
Use of venlafaxine, CYP2C9 variant alleles	4.52 (0.63 – 32.4)	
Interaction between fluvoxamine or venlafaxine and CYP2C9 genotype (wild type genotype / variant alleles)		0,49
No use of fluvoxamine or venlafaxine, CYP2C9 wild type genotype	1.00 reference	
No use of fluvoxamine or venlafaxine, CYP2C9 variant alleles	1.08(0.98 – 1.19)	
Use of fluvoxamine or venlafaxine, CYP2C9 wild type genotype	**2.10 (1.18 – 3.71)**	
Use of fluvoxamine or venlafaxine, CYP2C9 variant alleles	4.52 (0.63 – 32.3)	

Statistically significant values are printed in bold
a adjusted for age, sex, BMI, target INR and use of nonsteroidal anti-inflammatory drugs, ATC-group M01A and proton pump inhibitors (A02BC)

etine, citalopram, and sertraline reported no or only very small increases in prothrombin time which were not regarded to be of clinical relevance.[40-42]

A possible pharmacokinetic mechanism might be by competitive inhibition by SSRIs of the oxidative metabolism of coumarins via CYP2C9. Differences between the SSRIs could be explained through differences within the inhibitory potential on CYP2C9 which would prolongate the effectiveness of the coumarins and result in increased INR-measures. However, there is no consistent classification of the SSRIs concerning their inhibitory potential on CYP2C9. Fluvoxamine and fluoxetine have been reported to inhibit CYP2C9 most strongly within the SSRIs in one study[43], another mentioned fluoxetine, fluvoxamine and paroxetine with the highest potential for CYP2C9 interactions and had insufficient data to make a prediction for venlafaxine[44], whereas a Dutch textbook on drug information only described CYP2C9 inhibition for fluvoxamine and sertraline but not for the other SSRIs or venlafaxine.[26] For the coumarins, CYP2C9 is the principal catalyst for the (S-) enantiomeric forms of warfarin and acenocoumarol.[45] In general, the (S-)isomers of the coumarins are more effective. However, for acenocoumarol, the (S-)form has an extremely short half live of 2 hours and acenocoumarol effects mainly depend on the (R-)enantiomeric form of which about 60% is metabolized

via CYP2C9. Thus interactions via CYP2C9 inhibition may be more relevant for warfarin than for acenocoumarol. From our analysis, no effect modification of a *CYP2C9* variant allele was detected on the effect of fluvoxamine or venlafaxine on INR increase within acenocoumarol users. Possibly, more cases are needed to attain enough power for a genotype-stratified analysis that can confirm the effect of the *CYP2C9* genotypes on a pharmacokinetic interaction between acenocoumarol and SSRIs.

Besides a pharmacokinetic interaction that leads to increased INR levels, SSRIs deplete platelet serotonin levels and impair platelet aggregation with a subsequent increase of the risk of haemorrhage.[12-17] This risk might be higher for fluoxetine, paroxetine and sertraline as they inhibit the reuptake of serotonin stronger than citalopram and fluvoxamine. Unfortunately, in our study population of 2,755 acenocoumarol users and within these 222 SSRI users, we did not have enough bleeding events for stratified analysis for the separate SSRIs to prove this assumption. Other studies on warfarin reported no risk increase for the combination with SSRIs on upper gastrointestinal bleedings [23, 24, 46]. For acenocoumarol one case-control study found a risk increase on non gastrointestinal bleedings in combination with SSRIs[22]. However, this study did not stratify for the separate SSRIs. For a final estimation concerning the safety of SSRIs in combination with coumarins, the risks for bleedings with a specific SSRI should be studied.

Our study is the first observational cohort study on a risk increase of overanticoagulation with acenocoumarol by co-medication of all SSRIs during acenocoumarol maintenance treatment. We consider the chance of bias and confounding de to study design as negligible. First, the Rotterdam Study is a prospective cohort study and the regional anticoagulation clinic covered a complete area of more than one million inhabitants in the Rotterdam area. Consequently, everyone who is treated with a coumarin anticoagulant as an outpatient will be registered as such and selection bias is unlikely. Second, all medication use of all subjects was almost completely covered by the pharmacy data we retrieved and SSRIs were only available on prescription via pharmacies. Any lack of compliance would move our results into the direction of the null hypothesis, and would tend to make our results conservative. Third, we followed the cohort members during their whole period of acenocoumarol maintenance therapy and compared cases to all other cohort members available at the index dates for SSRI exposure. This analysis made use of all data available and adjusted for time varying effects.

However, in observational studies there is always a risk of confounding by indication. We therefore performed the same analysis for nortriptyline and mirtazapine, two antidepressants used for the same indication but not known for inhibitory potential of CYP2C9. As expected, we did not find an association for these drugs with an increased risk on achieving INR ≥6 during acenocoumarol treatment.

In conclusion, in this population-based cohort study among outpatients of an anticoagulation clinic using acenocoumarol for maintenance treatment, fluvoxamine and venlafaxine were associated with a more than double risk on an INR≥6. Fluoxetine, citalopram, paroxetine,

sertraline and escitalopram had no association with overanticoagulation during acenocoumarol maintenance treatment. If the latter drugs increase the risk of bleeding, it is likely that another mechanism is causative.

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Chapter 5

General discussion

Introduction

Epidemiology is usually defined as "the study of the distribution, determinants and control of health related states and events in populations".[1] *Pharmaco*-epidemiology focuses on drugs as determinants or risk factors for health outcomes.[2] If a drug increases the risk of a disease, this is usually called an adverse drug event, and the decrease of the disease risk is regarded as therapeutic drug effect. In clinical practice, drug response can differ considerably between patients: some patients do not respond at all to certain drug treatments whereas others respond too strongly or have serious adverse effects. Consequently, the precise dose required to achieve a response can vary considerably between patients. Commonly according to a 'one dose fits all' paradigm, patients start with standard dosages and if necessary treatment is accustomed to individual response. An example of a drug with which 'one dose fits all' gives problems is the antimalarial agent mefloquine of which a weekly dose of 250 mg caused neuropsychiatric adverse effects in vulnerable individuals.[3] Therefore this approach can put patients at considerable risks of serious adverse effects, further illustrated by the finding that approximately 6% of unplanned hospital admissions were due to medication of which potentially half could have been avoided if individual risk factors of the patients had been recognized and dealt with in advance.[4, 5] The contribution of genetic variation to these individual risk factors is the topic of interest in pharmacogenetics.[6] In this thesis, the influences of genetic variability in the recently detected *VKORC1* gene on aortic calcification and blood coagulation as well as coumarin effectiveness were studied. As part of that, also hypothesis-free genome-wide associations with coumarin effectiveness were investigated. We focused on treatment with coumarins because this is a drug group with established clinical use but with a small therapeutic range and a potentially life-threatening bleeding risk. Coumarin anticoagulants are among the most important causes of iatrogenic hospital admissions [4, 5]. The limits and merits of the individual studies underlying this thesis have been discussed in the previous chapters. In this chapter we will discuss our main findings and put them into the broader context of potential clinical implications and further research.

Main findings

Mendelian randomization to VKORC1 genotypes

In 2004 the gene encoding the molecular target of coumarin anticoagulants, vitamin K epoxide reductase complex subunit 1 (VKORC1) was identified.[7, 8] Mutations in this gene lead to deficiency of the vitamin K dependent proteins: the coagulation factors and protein C, S, Z and Matrix Gla protein (MGP). The dependency of these proteins on the VKORC1 enzyme differs within tissues. In the liver, besides VKORC1 another enzyme is involved in the activation of coagulation factors, in extra hepatic tissues however, the activation of MGP is fully

dependent on VKOR activity. [9, 10]. The key function of MGP is to inhibit tissue calcification. [11] As genetic variation in the *VKORC1* gene leads to a lifelong diminished functionality of the VKORC1 enzyme, we assumed that the effects of the variant genotype should become visible in vascular calcification. As genotypes are assigned randomly when passed from parents to offspring during meiosis, the distribution of genotypes in a population follows a pattern which is called "Mendelian randomization". Consequently, the population genotype distribution is supposed to be unrelated to the confounders that typically plague observational studies. We were able to show that the presence of the variant T-allele of the VKORC1 1173C>T SNP (rs9934438) was associated with a significantly increased risk of aortic calcification by 21%. Expectedly, this risk increase was relatively small as it is unlikely that a common genetic variation in the population found at one locus would cause a high risk of clinical relevant outcomes with a disadvantage in survival. Potential clinical implications of our finding are not clear yet as vascular calcification might lead to plaque erosion[12] as well as to plaque stabilization[13]. One study showed that this variant allele of *VKORC1* was less prevalent in patients with venous thromboembolism[14], but further studies are needed to elucidate its association with pulmonary embolism, myocardial infarction and stroke.

Influences of VKORC1 and CYP2C9 polymorphisms on coumarin effectiveness

After the identification of the *VKORC1* gene [7, 8] and the detection of single nucleotide polymorphisms which were able to explain variation in the activity of the coded enzyme, many studies were performed on the genetic influence of polymorphisms in this gene on treatment with warfarin [15-39] and fewer with acenocoumarol [40-43] and phenprocoumon.[44-46]. The influence of reduced VKORC1 activity on interpersonal dosage variation was comparable for each coumarin with 26-28%.[45, 47-49] This agreement was expected as the three coumarins operate pharmacodynamically via inhibition of the VKORC1 protein. The effects of *CYP2C9* polymorphisms on coumarin dosage were less and varied due to different pharmacokinetics from 12% for warfarin[48], 6% for acenocoumarol[49], to a negligible influence on phenprocoumon dosage [50].

In chapter 3, we described the results from our candidate gene studies with polymorphisms in the *VKORC1* and the *CYP2C9* gene on acenocoumarol and phenprocoumon dosage. In agreement with the other studies, *VKORC1* polymorphisms explained 28% of acenocoumarol and 26% of phenprocoumon dosage variation; *CYP2C9* polymorphisms explained 5.8% of variation of acenocoumarol and 2.9% of phenprocoumon dosage. In chapter 3.1, we further showed that variance in the *VKORC1* and the *CYP2C9* gene significantly increased the risk of overanticoagulation during the initiation period (OR per *VKORC1* variant allele: 1.57; 95% CI 1.19–2.07) and OR per *CYP2C9**2 or *3 variant alleles: 1.28; 95% CI 1.04–1.56). Each *VKORC1* variant allele was associated with a decrease in acenocoumarol dosage by 5.09 mg/week and for each *CYP2C9* *2 or *3 allele dosage decreased by 1.80 mg/week. In chapter 3.3, we described the results of genetic variation in these two genes on phenprocoumon

maintenance dosage: per *VKORC1* variant allele phenprocoumon dosage decreased by 4.8 mg/week and per *CYP2C9* variant allele by 2.2 mg/week. It has to be noted that in the analysis for acenocoumarol we used the combined genotype of *CYP2C9*2* and *3 with in total 6 composed genotypes (*1/*1, *1/*2, *1/*3, *2/*2, *2/*3, *3/*3) whereas for phenprocoumon we represented CYP2C9 activity by one SNP with 3 genotypes (wild type genotype, heterozygous for a variant allele and homozygous for the variant allele). Consequently, the stronger influence of polymorphisms in *CYP2C9* on acenocoumarol was visible in 9 mg/week (divided in dosage changes of 1.8 mg/week for five variant genotypes), compared to 4.4 mg/week for phenprocoumon (divided in dosage changes of 2.2 mg/week for two variant genotypes). We could show that the dosage variation due to reduced activity in the *VKORC1* and the *CYP2C9* gene added up without multiplicative modification of the effect measure. This independency of the effects from genetic variation within the *VKORC1* and *CYP2C9* genes was expected as etiologically the mechanisms of operation differ between the two genes and linkage disequilibrium is unlikely due to their location on different chromosomes.

No further relevant genes related with coumarin effectiveness from GWAS

Until now two GWAS were performed on warfarin effectiveness.[47, 48] The first warfarin GWAS by Cooper et al involved 181 patients and found a significant association for the *VKROC1* gene only.[47] *CYP2C9* did not show genome-wide significance but further genotyping in a replication cohort confirmed the strong effect for *VKORC1* and the weaker *CYP2C9* effect.[47] In a second GWAS on warfarin effectiveness by Takeuchi et al with 1,053 subjects involved, both *VKORC1* and *CYP2C9* reached genome-wide significance and after adjustment for their influences, the weaker association of *CYP4F2* was detected.[48] Our GWAS with 1,525 subjects, described in chapter 3.2, confirmed these results for acenocoumarol with identification of SNPs in the *VKORC1*, *CYP2C9* and *CYP4F2* genes and added a novel feature by detecting a genome-wide significant association of the *CYP2C18* gene. As no substrates for CYP2C18 have so far been identified, the association may relate to the adjacent *CYP2C19* gene, which codes for an enzyme that metabolizes (R-)acenocoumarol for 20%.[51] In a review, these three GWAS on coumarin dose requirement were considered valuable to confirm findings from previous candidate-gene studies.[52] However, they have not detected novel genetic factors that substantially affect coumarin maintenance dosage apart from a small additional contribution of *CYP2C19* for acenocoumarol dose.[52] In chapter 3.3 we described a GWAS on phenprocoumon maintenance dosage in 202 subjects. This number was sufficient to confirm the major influence of SNPs in the *VKORC1* gene, but we did not have enough power to detect weaker associations.

All present evidence clearly suggests that effectiveness of all three coumarins mainly depends on genetic variation of *VKORC1* and *CYP2C9*. With large patient cohorts polymorphisms in *CYP4F2* could be detected that accounted for 1% of coumarin dosage variation. For acenocoumarol also polymorphisms in *CYP2C18* had a similar influence. It is unlikely that there are other genes with influence on coumarin effectiveness stronger than that.

Drug-drug interactions with acenocoumarol

In chapter 4, we described the risk of overanticoagulation during acenocoumarol maintenance treatment by co-medication with proton pump inhibitors, PPIs, (chapter 4.1) and by selective serotonin reuptake inhibitors, SSRIs (chapter 4.2). We took an INR value of six and greater as outcome for overanticoagulation as the risk of bleeding steeply increases from this point.[53] Within the PPIs, co-medication with esomeprazole doubled the risk of an INR≥6 (HR 1.99; 95% CI 1.55–2.55) and lansoprazole was associated with a 50% risk increase (HR 1.49; 95% CI 1.05–2.10). The risk estimates for co-medication with omeprazole, pantoprazole and rabeprazole were lower and did not reach statistical significance. For the SSRIs, combination of acenocoumarol with fluvoxamine and venlafaxine was associated with a more than double risk on an INR≥6, for fluvoxamine with HR 2.63; 95% CI 1.49–4.66 and for venlafaxine with HR 2.19; 95% CI 1.21-3.99. The other SSRIs did not increase the risk of overanticoagulation with acenocoumarol. For drugs from both drug groups, case reports had signaled a possible drug-drug interaction with coumarins.[54-64] The Dutch Federation of Anticoagulation Clinics had added the whole group of SSRIs, and as of January 2010 (es)omeprazole, to the list of potentially interacting drugs with coumarins.[65] From our results, the guidelines of the Dutch Federation of Anticoagulation Clinics for extra INR monitoring with PPIs should be expanded with lansoprazole. For the SSRIs extra INR monitoring seems necessary for fluvoxamine and venlafaxine.

Interethnic genetic variation

Our findings described above all concern Caucasian populations with a prevalence of *VKORC1* variant alleles of approximately 40%. Prevalence of *VKORC1* variant alleles however is much higher in Asians with 95% and much lower in African Americans with 14% of the population.[66] The most frequent genotype ('major allele') in a population is called 'wild type genotype' for those genotypes with two alleles. For *VKORC1*, the most frequent genotype in Asians is the variant genotype in Caucasians. Due to the higher prevalence of the less active VKORC1 protein, Asians are more sensitive to coumarins than Caucasians. In African Americans, however, reduced activity of VKORC1 is less prevalent and thus on average higher coumarin dosages are needed in this population than in Caucasians.[15, 66, 67] Related to the prevalence of the VKORC1 genotypes, coumarin dosages for other ethnic groups are in the range between Asians and African American subjects.[28, 30, 35, 67] Consequently, the ability to predict the risk of overanticoagulation varies across races as some studies showed that *VKORC1* polymorphisms were less useful in predicting overanticoagulation in African Americans.[68, 69]

The prevalence of *CYP2C9*2* and *3 variant alleles also differs according to race. Polymorphisms in this gene were more common in Caucasians (13% for *2 and 7% for *3[70])

compared to African Americans (2.9% for *2 and 2.0% for *3[71]) and Asians (0.1% for *2 and 1.1-3.6% for *3[71]). Therefore, dosage algorithms based on *VKORC1* and *CYP2C9* genotypes have to be calculated and validated separately in the different populations.[72]

Methodological considerations

Adequate sample size for GWAs

In our GWAS on phenprocoumon (chapter 3.3) we were able to detect associations with genetic variation in the *VKORC1* gene but not with the *CYP2C9* and *CYP4F2* genes. However, with the candidate gene approach influence of genetic variation in the *CYP2C9* and *CYP4F2* genes was detected. With the 202 subjects included in our study, numbers were too low to trace weaker sources of variation in these genes, let alone to find new ones. The power to trace genome wide significantly dosage changes of 1–2 mg/week in genes with a variant allele frequency of 20% in a population of 200 subjects was only 2%. In our acenocoumarol study with 1500 subjects we had 80% power to detect SNPs with an allele frequency of 10% at a genome wide significance threshold of 5×10^{-8} associated with dosage changes of 4-5 mg/week. In comparison, to find under these conditions a SNP with an allele frequency of 10% to detect dosage changes of 1-2 mg/week would require 3.500 subjects. To detect milder effects of dosage changes of 0.5 mg/week with a frequency of the variant allele of 5% at genome wide significance with a power of 80% would require a study population of 15,000 subjects. Therefore the GWAS on coumarins performed so far were able to detect relatively large effects for frequent genetic variation. As the dosing algorithms built with this information still only detect half of the interpersonal variance of coumarin dosage, there may still be a substantial contribution of other currently unknown genetic variant alleles.

Repeated measurement analysis for recurrent events

Associations between an exposure and a certain event are usually studied with logistic regression analysis. With the Cox proportional hazards model, the time to event is also taken into account. However, these models require independence of events and can only deal with one outcome per person. Bleedings and events of INR ≥6.0, however, can occur repeatedly in the same person during the period of coumarin treatment. To make efficient use of all available information, advanced analysis techniques can be applied. With generalised estimating techniques (GEE) it is possible to take the dependency of observations within one person into account by adjusting for recurrent events within the same subject. This is done by adding a correlation matrix to the regression model that estimates the correlation between outcomes at different time points within one patient.[73] Logistic GEE analysis enabled us to analyze the whole pattern of all recurrent INR ≥6 events during acenocoumarol maintenance therapy in chapter 3.1. Another method is the Andersen Gill (AG) analysis, which is an extension of the

Cox proportional hazards model and allowed to study time dependent associations of repeated events. With conventional Cox-regression models cases are censored after the first event and thus further events are not taken into account. In chapter 4, we studied possible influence of co-medication on elevated INR values during acenocoumarol treatment. The exposure to co-medication could vary over time. With conventional Cox regression analysis we would have missed relevant information as due to censoring at the first event, the information from later events is missed. When a certain combination of exposures is relatively rare, censoring at the first event may substantially reduce power. Application of the AG analysis increased our power to detect the effects of co-medication. The results from this analysis were comparable to results from conventional Cox regression analysis in order of magnitude, but confidence intervals became smaller. Apparently, repeated measurements techniques can be useful for the analysis of repeated events to time dependent exposure variables and interacting drugs.

From pharmacogenetics to individualized drug therapy

Coumarins seem to be ideal candidates for the application of genetic-guided medicine as their effectiveness mainly depends on genetic variation in two genes, and because they are widely prescribed and have to be handled carefully within a narrow therapeutic range to prevent serious adverse effects.[74] In 2007, the Food and Drug Administration extended the product labelling information of warfarin with the notification that genetic data might be relevant for prescribing decisions.[75] However, this has not led to a change in guidelines by specialist societies such as the American College of Chest Physicians [76] because of lack of randomized data to provide evidence of the benefits and risks of dosing algorithms including genotype information.[77-79] Up to now, dosing algorithms have been developed that combine age, sex, body weight and co-medication with genetic information and were made online available.[74] However, their use in clinical practice is still limited as their application is complicated.[70] First, assessments on the utility of pharmacogenetic determined coumarin dosage algorithms showed that the accuracy of predicted values was highest in the categories with the most extreme dosages.[80] Warfarin-related genotyping was not cost-effective in all users and might be only useful in those with a high risk for haemorrhage at the start of therapy.[81] Currently, two trials are ongoing to prove the clinical benefit of personalized dosage prediction in terms of safety and clinical utility. One is a multicentre study in seven European countries, the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial [82], the other study is the NIH-funded Clarification of Optimal Anticoagulant through Genetics (COAG) trial in the US [83]. It has to be noted that the situation in the Netherlands differs from the other countries as here anticoagulation therapy with coumarins is monitored by specialised clinics. Therefore possible incremental benefits of personalized dosage prediction found by these studies have to be specifically evaluated for the Dutch situation.

If these studies prove that dosing according to a genotype-guided algorithm will increase the safety and efficacy of oral anticoagulation therapy at acceptable cost-effectiveness, this could lead to pharmacogenetic dosing at initiation of coumarin therapy in hospitals as well as in primary care.[82]

A future role for pharmacists

In the Netherlands, anticoagulation clinics have a major task in managing anticoagulation treatment. In order to keep the INR outcomes within the target range, extra monitoring is necessary if medication is added that affects response to coumarins. Community and hospital pharmacists have the responsibility to search for alternatives available that do not interfere with coumarins. If alternatives are not an option, pharmacists have to inform the patient's anticoagulation clinic to warrant adequate adjustment of coumarin dosage during additional use of the interacting medication. Care of pharmacists for coumarin users was measured in a set of quality indicators for community pharmacies, developed by the Drug Safety Unit of the Inspectorate for Health Care (IGZ) and the Royal Dutch Association for the Advancement of Pharmacy (KNMP).[84] Evaluation from the measurement over the year 2009 showed that all 1,830 community pharmacies which completed the questionnaire had working procedures implemented to inform anticoagulation clinics, patients or prescribers about the necessity of extra INR monitoring. Ninety-four percent of community pharmacies in general directly informed the anticoagulation clinics when interacting co-medication was dispensed. This showed that pharmaceutical care concerning coumarin treatment is broadly implemented in daily practice. Another quality indicator from this set calculated patients with probable interactions between coumarins and miconazol as percentage of all coumarin users. As this drug combination is hazardous and alternatives for miconazol are available that do not interact with coumarin effectiveness, this combination has to be absolutely avoided. The prevalence of this combination was estimated by the aid of dispensing data from community pharmacies which were collected by the Dutch Foundation of Pharmaceutical Statistics (SFK). As a result in 81% of the community pharmacies, no patients on this combination were detected, 14% of the pharmacies had a maximum of 1% of coumarin users potentially exposed to this combination and 5% of pharmacies had higher percentages. Thus the majority of community pharmacies efficiently handled coumarin-drug interactions, but this still has to be improved in a small group of pharmacies.

The role of the pharmacist in the safe use of coumarins could extend substantially in future with the availability of genetic information. Herewith, pharmacists could give individualized advice on drug choice, interactions and contra-indications based on personalized risk/ benefit considerations. In anticipation of this development, the Drug Information Centre of the Royal Dutch Association for the Advancement of Pharmacy as of 2006 started in supplying infor-

mation on genetic variation that can influence drug effectiveness.[85] Currently information on the polymorphisms in the following genes is available *CYP2D6, factor V Leiden, CYP2C9, CYP2C19, VKORC1,* thiopurine methyltransferase (*TPMTI*) and UDP-glucuronosyltransferase (*UGT1A1*). For the drugs influenced by genetic variation, in future this information has to be linked in pharmacies' and prescribers' computer systems to perform "individualized medication surveillance" prior to dispensing. In this way, besides a personalized dosage advise genotypes could be taken into account to classify the relevance of a drug/drug interaction. Consequently, signaling of such an interaction might then become more specific and depend on the question whether the patient is a poor, normal or extensive metabolizer. At this moment, co-medication of coumarins with co-trimoxazol is acceptable with additional monitoring but should preferably be avoided.[86] With information of CYP2C9 activity available, co-medication with co-trimoxazol might be acceptable in patients with normal CYP2C9 enzyme activity in combination with extra INR-monitoring and would thus not be 'flagged' by the system. For persons with *CYP2C9* variant alleles, however, there should be an alert for this interaction because in these patients the combination potentially introduces an unacceptable risk of overanticoagulation and bleeding that has to be avoided.

Future research

At the moment, we are still a long way from the day when a patient presents a DNA "chip", a key-chain tag bearing the patients electronic health record, to get a dose of personalized medicine.[6] However, expectations in the US on economic effectiveness from standard use of genetic testing in general practice to determine initial warfarin dose are high with potentially 85,000 serious bleeds and 17,000 strokes to be avoided annually.[87] Future research has to show if these predictions are realistic. Possibly the ongoing studies on genetic forecasting of coumarin dosage in Europe and the US might accelerate developments to personalized medicine if they are able to show that genotype-guided dosing can improve safe use of coumarins at expenses that are outweighed by the benefits of saving costs for the treatment of serious adverse effects. Developments in information technology and broad social acceptance of determining and sharing genetic information, might bring the "DNA key chain" closer to reality. This genetic information might then be used to predict a patient's drug response and by this to optimize treatment choice for the individual patient: For clopidogrel in March 2010, the US Food and Drug Administration approved a new label with a "boxed warning" describing the diminished effectiveness of the standard drug dosing in individuals with impaired metabolic function (so-called poor metabolizers) based on their *CYP2C19* genotype.[88] As genetic tests are available, patients with genetic polymorphisms could be identified and alternative treatment strategies, either higher clopidogrel dosing regimens or the use of other antiplatelet agents, should be considered in those patients. However, at the

moment, we are still waiting for the results of the ongoing trials and therefore clopidogrel therapy and *CYP2C19* genetic testing remain a limited piece of the overall therapeutic puzzle of individualized drug therapy.[89] For coumarins, results from cost-benefit studies with direct comparison between effectiveness of coumarins and the direct thrombin inhibitors in daily practice might show whether subjects with variant alleles in the *VKORC1* as well as in the *CYP2C9* genes should get a direct thrombin inhibitor such as dabigatran instead of a coumarin anticoagulant. In these persons, stable anticoagulation with coumarins is hard to achieve and the increased risk of life-threatening bleedings in these patients might possibly outweigh the still unknown risks of direct thrombin inhibitors in long term treatment. When the results of ongoing research can be translated into clear guidelines, pharmacists could apply these new insights together with other patient related factors (e.g. age, sex, co-medication, co-morbidity, allergic reactions and environmental agents such as smoking and alcohol) and drug related factors (e.g. pharmacokinetics, pharmacodynamics, adverse effects, interactions, contra-indications) into individualized drug choice and drug monitoring.

Conclusion

Despite all difficulties to handle coumarins in clinical practice, these drugs will keep playing an important role in anticoagulation therapy until newer drugs have proven their superiority.

Due to their marked influence on coumarin response, the *VKORC1* and *CYP2C9* genotypes are suitable for an individualized medicine approach in anticoagulation therapy with coumarins. In the future pharmacists, could play an important role in " individualized medication surveillance" if ongoing research proves the benefits of pharmacogenetic testing and if pharmacists succeed in combining genetic information with other patient and drug related factors to improve medication effectiveness and medication safety.

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Chapter 6

Summary

Chapter 6.1

Summary

Coumarins are drugs with established value in clinical use but with a small therapeutic range and a potentially life-threatening bleeding risk. As warfarin is the coumarin used most frequently worldwide, the majority of studies is published on this anticoagulant. Due to different pharmacokinetic properties of each drug, these results may not be applicable to acenocoumarol and phenprocoumon, the coumarins which are predominantly used in the Netherlands. The studies described in this thesis are a contribution to the ongoing research in finding the right balance between benefits and risks of coumarin treatment. They were all performed within the Rotterdam Study, a large prospective population-based cohort study to assess the prevalence, incidence, and determinants of diseases in the elderly.

In **Chapter 1** a general introduction to anticoagulation therapy, the pharmacodynamic and pharmacokinetic characteristics of coumarins, candidate gene and genome-wide association studies (GWAS) is given. In **chapter 2** we investigated whether genetic variation in the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) with a lifelong reduced activity of the encoded enzyme was associated with increased calcification of the aortic far wall. We found a statistically significant risk increase of 21% (95% CI 2 to 43%) on aortic calcification in persons with at least one variant allele compared to those with the wild type genotype. In **chapter 3.1** we studied the effects of variant alleles in the *VKORC1* and the cytochrome P450 isoform 2C9 (*CYP2C9*) genes on anticoagulation therapy with acenocoumarol during the initial treatment period. With the standard dosage scheme of 6, 4 and 2 mg acenocoumarol during the first three days of treatment initiation, at the first INR measurement each *VKORC1* variant allele was significantly associated with a 85% risk increase of severe overanticoagulation. During the first six weeks of treatment, persons with variant alleles in both genes remained at an increased risk for severe overanticoagulation although dosage was titrated during this period by INR outcomes to a personal maintenance dosage. The dosage for acenocoumarol maintenance therapy was decreased for each *VKORC1* variant allele by 5.1 mg/week and for each *CYP2C9* variant allele by 1.8 mg/week. **Chapter 3.2** described the results of a GWAS on acenocoumarol maintenance dosage. Inter personal dosage variation mainly depended on polymorphisms in the *VKORC1* (28%) and *CYP2C9* (6%) genes. Genetic variation in *CYP4F2* and *CYP2C18* contributed with 1% each. A model with age, sex, body mass index, target INR and one polymorphism within each of the *VKORC1, CYP2C9, CYP4F2* and *CYP2C18* genes explained 48.8% of variation in acenocoumarol maintenance dosage. In **chapter 3.3** we studied genetic influences on phenprocoumon maintenance dosage. We used a candidate gene approach to study the association with single nucleotide polymorphisms (SNPs) in *VKORC1, CYP2C9* and *CYP4F2*, using SNPs we had already identified in the GWAS on acenocoumarol. We furthermore assessed associations with apolipoprotein E (*ApoE*) as genotypes in this gene were shown earlier to affect acenocoumarol dosage. In a GWAS, we studied these and more genetic associations with phenprocoumon maintenance dosage. We confirmed earlier findings that phenprocoumon maintenance dosage mainly depended on polymorphisms in the *VKORC1* gene. Genetic variation within *CYP2C9* and *CYP4F2* genes were of modest relevance. A model

with age, sex, body mass index, target INR and one polymorphism within each of the *VKORC1*, *CYP2C9* and *CYP4F2* genes explained 46.0% of variation in phenprocoumon maintenance dosage. In **chapter 4.1** we investigated the effects of co-medication with proton pump inhibitors (PPIs) on INR≥6 values during acenocoumarol maintenance treatment. Until April 2009, nine case-reports were collected by the Netherlands' national pharmacovigilance center (LAREB) for omeprazole and seven for esomeprazole. Co-medication with esomeprazole was associated with a significant double risk of overanticoagulation and lansoprazole increased this risk by 50%. There were also risk increases for the other PPIs, but these increases were lower and statistically non-significant. In **chapter 4.2** we studied the effects of co-medication with selective serotonin reuptake inhibitors (SSRIs) on INR≥6 values during maintenance therapy with acenocoumarol. Combinations with fluvoxamine and venlafaxine more than doubled the risk for overanticoagulation (for fluvoxamine HR=2.63; 95% CI 1.49–4.66 and for venlafaxine HR= 2.19; 95% CI 1.21–3.99). Co-medication with the other SSRIs was not associated with INR values of six and higher. The general discussion in **chapter 5** summarized the main findings and discussed some methodological issues. Implications of our findings on the treatment with coumarins and the role of the pharmacist together with recommendations for future research were given.

Chapter 6.2

Samenvatting

Coumarine anticoagulantia hebben hun waarde bewezen in de klinische praktijk. Zij hebben echter een smalle therapeutische breedte waardoor een behandeling met deze geneesmiddelen kan leiden tot levensbedreigende bloedingen. Warfarine wordt uit de groep van coumarines wereldwijd het meest voorgeschreven, en daarom zijn over dit middel de meeste studies beschikbaar. Aangezien de coumarines onderling verschillen in hun farmacokinetische eigenschappen laten zich de bevindingen voor warfarine niet zonder meer vertalen naar acenocoumarol en fenprocoumon, de coumarines die in Nederland voornamelijk worden gebruikt. De artikelen in dit proefschrift leveren een bijdrage aan het lopende onderzoek naar de optimale verhouding tussen nut en risico bij de behandeling met deze geneesmiddelen. Het hier beschreven onderzoek is verricht binnen de Rotterdam Studie, een groot prospectief bevolkingsonderzoek naar de frequentie en de oorzaken van chronische ziekten bij ouderen.

Hoofdstuk 1 geeft een algemene introductie over de bloedstolling en de therapie met geneesmiddelen voor antistolling, de farmacodynamiek en farmacokinetiek van de coumarines en de bijzonderheden van onderzoeken naar associaties met kandidaat genen en met genen uit het hele genoom. In **hoofdstuk 2** onderzochten wij of een genetische variatie in het vitamine K epoxide reductase complex subeenheid 1 (*VKORC1*), met als gevolg hiervan een levenslang verlaagde enzymactiviteit, in relatie stond met verkalking van de aorta. Wij vonden een met 21% statistisch significant verhoogd risico op aorta verkalking in dragers van tenminste één variant allel in het *VKORC1* gen ten opzichte van personen met het meest voorkomende genotype. In **hoofdstuk 3.1** onderzochten wij de effecten van variant allelen in de *VKORC1* en de cytochrome P450 isoform 2C9 (*CYP2C9*) genen op de werkzaamheid van acenocoumarol tijdens de startfase van de therapie. De mate van de intensiteit van antistolling werd uitgedrukt in 'international normalised ratio' (INR), waarbij een uitkomst van 6 en hoger een sterk verhoogde kans op bloedingen geeft, de zogenoemde 'doorgeschoten' INR. De gebruikelijke standaarddosering met 6 mg (dag 1), 4 mg (dag 2) en 2 mg (dag 3) acenocoumarol gedurende de eerste drie dagen van de therapie verhoogde in dragers van elk extra *VKORC1* variant allel het risico op een doorgeschoten INR met 85%. Gedurende de eerste zes weken van de behandeling met acenocoumarol bleef het risico op doorgeschoten antistolling verhoogd in personen met variant allelen in beide genen ondanks het feit dat de dosering gedurende deze instellingsperiode met behulp van de INR uitkomsten en gewenste therapeutische 'target level' bijgesteld werd tot een individuele onderhoudsdosering. De uiteindelijke individuele onderhoudsdosering acenocoumarol was met 5.1 mg/week per variant allel in het *VKORC1* gen verlaagd, en per *CYP2C9* variant allel met 1.8 mg/week. **Hoofdstuk 3.2** schetst de invloed van genetische factoren op de onderhoudsdosering van acenocoumarol met behulp van een genoom-brede associatie (GWA) studie. GWA onderzoeken maken het mogelijk om naast de bekende factoren nieuwe genetische loci te identificeren die invloed hebben op de variatie van de acenocoumarol dosering tussen personen. Wij vonden dat de verschillen in acenocoumarol onderhoudsdosering vooral bepaald werden door polymorfismen in de genen *VKORC1* (met 28%) en *CYP2C9* (met 6%). Genetische variatie in *CYP4F2* en

CYP2C18 droeg hier voor ieder gen met 1% aan bij. Een wiskundig model opgebouwd uit de variabelen leeftijd, geslacht, body mass index, INR-streefwaarde en een polymorfisme binnen de *VKORC1, CYP2C9, CYP4F2* en *CYP2C18* genen kon 48.8% van de verschillen tussen personen voor de hoogte van acenocoumarol onderhoudsdosering verklaren. In **hoofdstuk 3.3** bestudeerden wij de genetische invloeden op de onderhoudsbehandeling met fenprocoumon. Met een 'kandidaat gen' studieopzet brachten wij de invloed op de onderhoudsdosering met fenprocoumon in kaart van uit onderzoek met warfarine en acenocoumarol bekende 'single nucleotide polymorphisms' (SNPs) binnen *VKORC1, CYP2C9, CYP4F2* en apolipoprotein E (*ApoE*). In een GWAS zochten wij verder naar mogelijke associaties met nieuwe genen. Wij konden bevestigen dat ook de onderhoudsdosering fenprocoumon vooral bepaald werd door variatie in het *VKORC1* gen. Daarnaast waren polymorfismen in *CYP2C9* en *CYP4F2* van kleiner belang. Een wiskundig model opgebouwd uit de variabelen leeftijd, geslacht, body mass index, INR-streefwaarde en een polymorfismen binnen de *VKORC1*, *CYP2C9* en *CYP4F2* genen kon 46.0% van de verschillen tussen personen voor de hoogte van phenprocoumon onderhoudsdosering verklaren. In **hoofdstuk 4.1** onderzochten wij de effecten van een behandeling met proton pomp remmers (PPIs) tijdens een onderhoudsbehandeling met acenocoumarol op het doorschieten van de INR. Aanleiding waren negen meldingen over het doorschieten van de INR tijdens acenocoumarol gebruik in combinatie met omeprazol en zeven meldingen voor de combinatie met esomeprazol die tot april 2009 door het Nederlandse bijwerkingen centrum LAREB verzameld waren. Wij vonden voor esomeprazol een verdubbeld risico op een INR≥6, en voor lansoprazol een risicoverhoging van 50%. Voor de andere PPIs vonden wij lagere risico's die overigens statistisch niet significant waren. In **hoofdstuk 4.2** bestudeerden wij de effecten van co-medicatie met selectieve serotonine heropname remmers (SSRIs) op INR waarden van 6 en hoger tijdens een onderhoudsbehandeling met acenocoumarol. Fluvoxamine en venlafaxine zorgden voor een meer dan verdubbeld risico op doorschieten tijdens een behandeling met acenocoumarol (voor fluvoxamine was het relatieve risico 2.63, 95% betrouwbaarheidsinterval (95% BI) 1.49–4.66, en voor venlafaxine 2.19, 95% BI 1.21–3.99). De overige SSRIs lieten geen verhoogd risico zien op een INR waarde van 6 en hoger tijdens behandeling met acenocoumarol. In de algemene discussie in **hoofdstuk 5** hebben wij de belangrijkste bevindingen van de studies in dit proefschrift en een aantal methodologische overwegingen hierbij besproken. Tevens schetsten wij de betekenis van onze bevindingen voor de behandeling met coumarines, de rol van de apotheker bij het veilige gebruik van deze geneesmiddelen, en gaven wij aanbevelingen voor verder onderzoek op dit gebied.

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Research skills and in-depth courses

2010	Introduction to Pharmacogenetics. International Conference on Pharmacoepidemiology and Therapeutic Risk in Brighton, United Kingdom
2006 – 2010	Research seminars, Departments of Epidemiology and Medical Informatics, Erasmus MC, Rotterdam, the Netherlands
2008	SNPs and Human Diseases, Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands
2008	Farmacogenetica en wat zult u er aan hebben? Stichting Post-Academisch Onderwijs Klinische Chemie en Laboratoriumgeneeskunde (PAOKC), Rotterdam, the Netherlands
2006 – 2008	Advanced topics in Pharmacoepidemiological methods, special skills workshops, International Conference on Pharmacoepidemiology and Therapeutic Risk
2007	Systematische reviews, theorie en praktijk, Postgraduate Master Programme in Epidemiology, Amsterdam, the Netherlands
2006	Multilevel analyse, Postgraduate Master Programme in Epidemiology, Amsterdam, the Netherlands
2006	Longitudinale data-analyse, Postgraduate Master Programme in Epidemiology, Amsterdam, the Netherlands

(Inter)national conference presentations

Oral presentations

2010	"Opvolgen van het Zwangerschap Preventie Programma voor isotretinoine, een retrospectieve cohortstudie bij vrouwen in de vruchtbare leeftijd in Nederland", symposium of Practice Research in Cooperation with Pharmacists (PRISMA) in Amersfoort, the Netherlands
2010	"Implementatie van Medicijngesprekken met behulp van de KNMP-projectondersteuning", symposium of Practice Research in Cooperation with Pharmacists (PRISMA) in Amersfoort, the Netherlands
2009	"A genome-wide association study of acenocoumarol maintenance dosage", Lecture at the meeting of the KNMP-department Leiden, in the Archeon, Alphen, the Netherlands
2009	"Do pregnancy prevention programs work in Dutch female isotretinoin users of reproductive age?" Dutch Medicines Days of symposium Figon, Dutch Association for Clinical Pharmacology in Lunteren, the Netherlands
2009	"A genome wide association study on acenocoumarol maintenance dosage", COEUR seminar, Erasmus MC, Rotterdam
2008	"The influence of reduced activity of VKORC1 and CYP2C9 genotypes on anticoagulation in initial treatment with acenocoumarol", Dutch Medicines Days of symposium Figon, Dutch Association for Clinical Pharmacology in Lunteren, the Netherlands
2008	"Hoe bruikbaar zijn voorschrijfindicatoren op basis van de DU90% methode voor het aantonen van verschillen in voorschrijfkwaliteit tussen de verschillende kwaliteitsniveaus van FTO-groepen?", symposium of Practice Research in Cooperation with Pharmacists (PRISMA) in Amersfoort, the Netherlands
2008	"Genotypes associated with reduced activity of *VKORC1* and *CYP2C9* and their modification of acenocoumarol anticoagulation during the initial treatment period", 24th International Society for Pharmacoepidemiology in Copenhagen, Denmark
2007	"Discontinuation of beta-blockers and the risk of myocardial infarction in the elderly", 8th conference of the European Association of Clinical Pharmacology and Therapeutics in Amsterdam, the Netherlands.

Poster presentations

2010	"Dependency of phenprocoumon dosage on polymorphisms in the *VKORC1*, *CYP2C9* and *CYP4F2* genes", 26th International Conference on Pharmacoepidemiology and Therapeutic Risk in Brighton, United Kingdom
2009	"Do pregnancy prevention programs work in Dutch female isotretinoin users of reproductive age?", 25th International Conference on Pharmacoepidemiology and Therapeutic Risk in Providence, United States of America

Lecturing

2009	"Use of dispensing data for quality indicators", workshops for community pharmacists for quality assessment in cooperation with the Stevenshof Insitute for Pharmacy Practice and Policy, Utrecht, the Netherlands
2006 – 2009	"Working with dispensing data for quality assessment and improvement", training courses within the programme on Pharmaceutical Care for community pharmacists within the registration program of the KNMP, Utrecht, the Netherlands
2007 – 2009	"Imrprovement of medication safety and compliance with the aid of dispensing data", participation in the teaching program for 2nd year medical students of the Vrije Universiteit, Amsterdam, the Netherlands in cooperation with the division Metamedica, VU Amsterdam and 60 community pharmacists, Amsterdam, the Netherlands
2008	"Optimalization of medication with dispensing data", workshop for pharmacy students, division of Pharmacoepidemiology & Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, the Netherlands

Supervising practicals

2006 – 2010	Data-analysis in pharmacoepidemiology, medical students, Prof. Dr. B.H.Ch. Stricker, Erasmus MC, Rotterdam, the Netherlands

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2006 – now	Referee activities for various (inter)national scientific journals

About the author

Martina Teichert was born on October 19th, 1962 in Hanau, Germany. In 1981, she completed the Gymnasium at the 'Gesamtschule Freigericht' in Somborn, and started in the same year to study German, English and pedagogy at the 'Eberhard Karls Universität' in Tübingen. In 1982, she started to study pharmacy at the 'Technische Universität Carolo Wilhelmina' in Braunschweig. She obtained her university degree in 1987 and received her pharmacist's degree in 1988. Until 1990 she worked as a pharmacist in part time in different community pharmacies and a hospital pharmacy. During 1988 and 1990, she studied economics at the 'Technische Universität Carolo Wilhelmina' in Braunschweig, and graduated as a 'Diplom-Wirtschaftspharmazeut' ('sehr gut') in 1991. In 1991, she moved to the Netherlands. Until 1997, she worked as a freelance journalist for several journals as 'Medizinische Monatsschrift für Pharmazeuten', 'Deutsche Apotheker Zeitung', 'Apotheken Praxis 'and the 'European Hospital'. From 1997 to 2000, she was employed as a pharmacist at the 'Boots Apotheek', Rotterdam in part time. In 1997, she founded her own company 'Pharmaceutical Project Management' (PPM) and performed several tasks: from 1997 until 2000 she built up and implemented a quality management system in the 11 Boots pharmacies in the Netherlands at that time; from 1998 until 2004, she gave trainings for community pharmacists for the development and implementation of quality management systems on behalf of the 'Institut für Kommunikation und Führung', (IKF) in Undeloh and in cooperation with the German pharmacists departments in Niedersachsen, Sachsen and Sachsen Anhalt; between 2000 and 2002, she worked for the Quality Institute of Pharmaceutical Care (QIPC) in Kampen, and developed and implemented a model for 'kwaliteitsjaarplannen en -jaarverslagen'. Since 2002, she is working for the 'Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie' (KNMP) in Den Haag (head Dr. F.J. van de Vaart). There she coordinates a multi-disciplinary team for the development and implementation of web based reports, quality indicators and training courses on the improvement and evaluation of pharmaceutical care in community pharmacies. In 2002, she started with the postgraduate Master Program in Epidemiology at the Faculty of Medicine at the 'Vrije Universiteit' in Amsterdam and obtained in 2005 a degree as a Master of Epidemiology. In 2006, she started the work described in this thesis for two days a week at the Pharmacoepidemiology Unit (head: Prof. Dr. B.H. Ch. Stricker). She has three sons, Olaf (19), Erik (18) and Christoph (16).