Towards optimal dosing of coumarin derivatives The role of pharmacogenetics

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Towards optimal dosing of coumarin derivatives: The role of pharmacogenetics

Op weg naar een optimale dosering van coumarine derivaten: De rol van farmacogenetica

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 10 april 2013 des middags te 4.15 uur

door

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Chapter 1

General introduction

From a deadly cow poison to effective human drugs

The history of coumarin derivatives goes back to the 1920's[1-4]. In New Dakota (USA) and Alberta (Canada) cattle died from fatal hemorrhages. These hemorrhages were either spontaneously or from injuries and interventions such as dehorning or castration. The veterinarians Schofield and Roderick concluded that these hemorrhages were caused by eating sweet clover (Melilotus albus & M. medicinalis) spoiled by an Aspergillus mold and therefore this disease was called "sweet clover disease". After eating the spoiled sweet clover, there was a reduction in coagulation in approximately 15 days, which resulted in internal hemorrhages that became fatal in 30 to 50 days -if no injury occurred in the meantime-. Schofield observed that no hemorrhage occurred if non-spoiled sweet clover or only the mold Aspergillus was eaten. He assumed that the mold activated the sweet clover to produce a toxic compound. In 1939, Karl Link was able to isolate the toxic compound 3,3'-methylenebis (4-hydroxycoumarin), or shortly dicumarol, from the sweet clover and in 1940, the chemical structure was discovered[5]. From 1940 to 1942, dicumarol was turned over to the clinicians. It was hypothesized that if dicumarol could cause hemorrhages in cattle, it might prevent thromboembolic events after surgery in patients if given in lower dosages. However, despite lectures and publications, clinicians refused to use dicumarol in practice; they regarded it as a dangerous drug. In the meantime, Link and colleagues synthesized new coumarin derivatives to be used for other purposes, like a rodent poison for which dicumarol was not potent enough. Derivative number 42 was synthesized in 1942-1943 by Ikawa and its anticoagulant activity was assessed by Scheel. This derivative was shown to be more potent than dicumarol and was initially promoted as rodent poison. They called it warfarin, which is a composition of "WARF" (Wisconsin Alumni Research Foundation, key funding of the research) and "arin" from coumarin. In April 1951, an army inductee attempted suicide by taking multiple doses of warfarin. Because of the time to onset of the anticoagulation effect, he had time to regret his action. He was admitted to the hospital and recovered after blood transfusions and large dosages of vitamin K. This event made clinicians think more positively about warfarin, since warfarin showed some advantages compared with dicumarol. For example, it is 5 to 10 times more potent, and it can be administered via several routes, shortly; it was easier to handle clinically. It was approved for human use in 1954 and made clinically available by the Endo Laboratories, Richmond Hill, NY. President Dwight Eisenhower was one of the first patients using warfarin.[1-4]

To date, warfarin is the most prescribed vitamin K-antagonist worldwide[2]. In continental Europe, phenprocoumon and acenocoumarol are the coumarins prescribed most often. The coumarins have been proven to be effective in prevention of

thromboembolic events for patients suffering from atrial fibrillation, with artificial heart valves, after surgery, and for the treatment of venous thromboembolism. The anticoagulant therapy with coumarins however is complex. Coumarins have a small therapeutic window and the required dose varies enormously both between and within patients[6, 7]. Patients are continuously balancing between underdosing, which introduces a risk of thromboembolic events, and overdosing, which increases the risk of a hemorrhage. Therefore, the use of coumarins is often associated with drug-related hospitalization[8-11].

From an empiric approach to personalized medicines

Even though coumarins are already on the market for decades, the number of publications on these compounds received a boost in the early nineties (Figure 1). In that period, pharmacogenetics gained increasing interest. Pharmacogenetics is the study of variations in DNA sequence as related to drug response[12]. A dosage of a drug or a drug itself might not be as effective or safe in one patient compared with another patient. Genotyping the patient might assist physicians to determine the individualized dose, or identify patients that might not benefit or have a higher risk of suffering from adverse drug reactions. For example, treatment with abacivir in HIV positive patients carrying a variant allele, HLA-B*57:01, is associated with an increased risk of hypersensitivity, which might become fatal after repeated dosing[13]. For patients, it would be ideal if they could receive the right dose or right prescription immediately. This would increase the effectiveness and safety of therapy. With help of the genetic profile of a patient, for some drugs it might be possible to explain the variable response in patients.

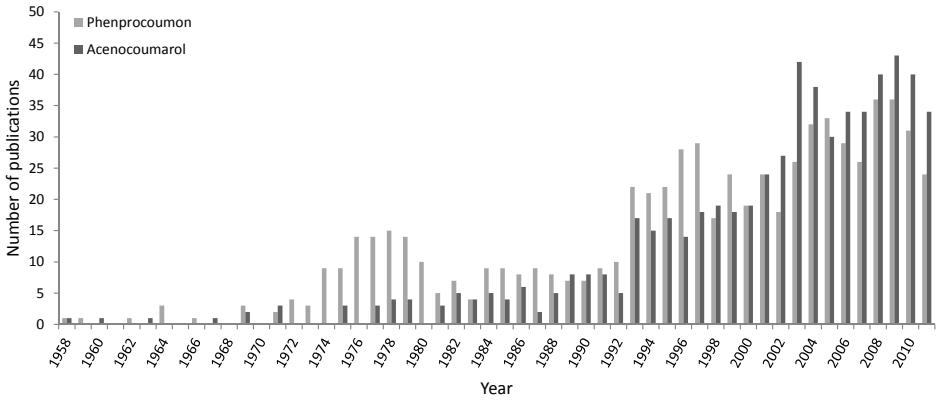

Figure 1. Number of publications for phenprocoumon and acenocoumarol through the years.

Pharmacogenetic studies were also performed for coumarins and so new information came available that explained the variation in the coumarin dose requirements and gave new insights in the anticoagulant therapy. In 1992, Rettie *et al.* identified CYP2C9 as the main metabolizing enzyme of warfarin[14] and in 1995, Furuya *et al.* showed that Single Nucleotide Polymorphisms (SNPs) in *CYP2C9* influence coumarin dose requirements[15]. Vitamin K epoxide reductase complex subunit 1 (VKORC1) was identified as the target enzyme for the coumarins in 2004[16, 17].

Objectives and outline of this thesis

The objective of this thesis is to gain insight in the individualization of oral anticoagulant therapy with coumarins.

In Part I, Chapter 2 we provide an overview of the pharmacogenetics of oral anticoagulant therapy.

In Part II, we discuss the development and usage of the phenprocoumon and acenocoumarol dose algorithms. In Chapter 3, the development of the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) loading and maintenance dose algorithms for phenprocoumon and acenocoumarol is described. We used information of over 1000 patients to develop dose algorithms containing information on patient characteristics (i.e. age, height, weight, sex, and amiodarone use) and pharmacogenetic data (*VKORC1* and *CYP2C9* genotypes). In Chapter 4, the validation of the acenocoumarol EU-PACT algorithm in the Rotterdam Study cohort is described. In Chapter 5, we describe the study design of the EU-PACT trial, that is still ongoing, and in which we investigate the algorithms that we have developed and that are described in Chapter 3. The primary aim of the EU-PACT trial is to investigate the added value of pretreatment genotyping on the percentage time spent within target International Normalized Ratio (INR) range.

In Part III, we discuss the effects of genetic variances and comedication use on the anticoagulant therapy. In Chapter 6, we describe the possible gene-gene interaction between *CYP2C9* and *VKORC1* genotypes affecting the anticoagulant effect of phenprocoumon and acenocoumarol. In Chapter 7, the effect of SNPs in *GATA-4* on the phenprocoumon and acenocoumarol maintenance dose is depicted. In Chapter 8, we studied the effect of SNPs in *CYP3A4* and *CYP4F2* on the phenprocoumon maintenance dose. In Chapter 9, we describe an evaluation of the effects of statin use on the acenocoumarol and phenprocoumon maintenance dose. In Chapter 10,

we provide a general discussion, putting our results in a broader perspective, elaborating on the challenges of implementation, summarizing the developments in oral anticoagulant therapy, and providing ideas for future research.

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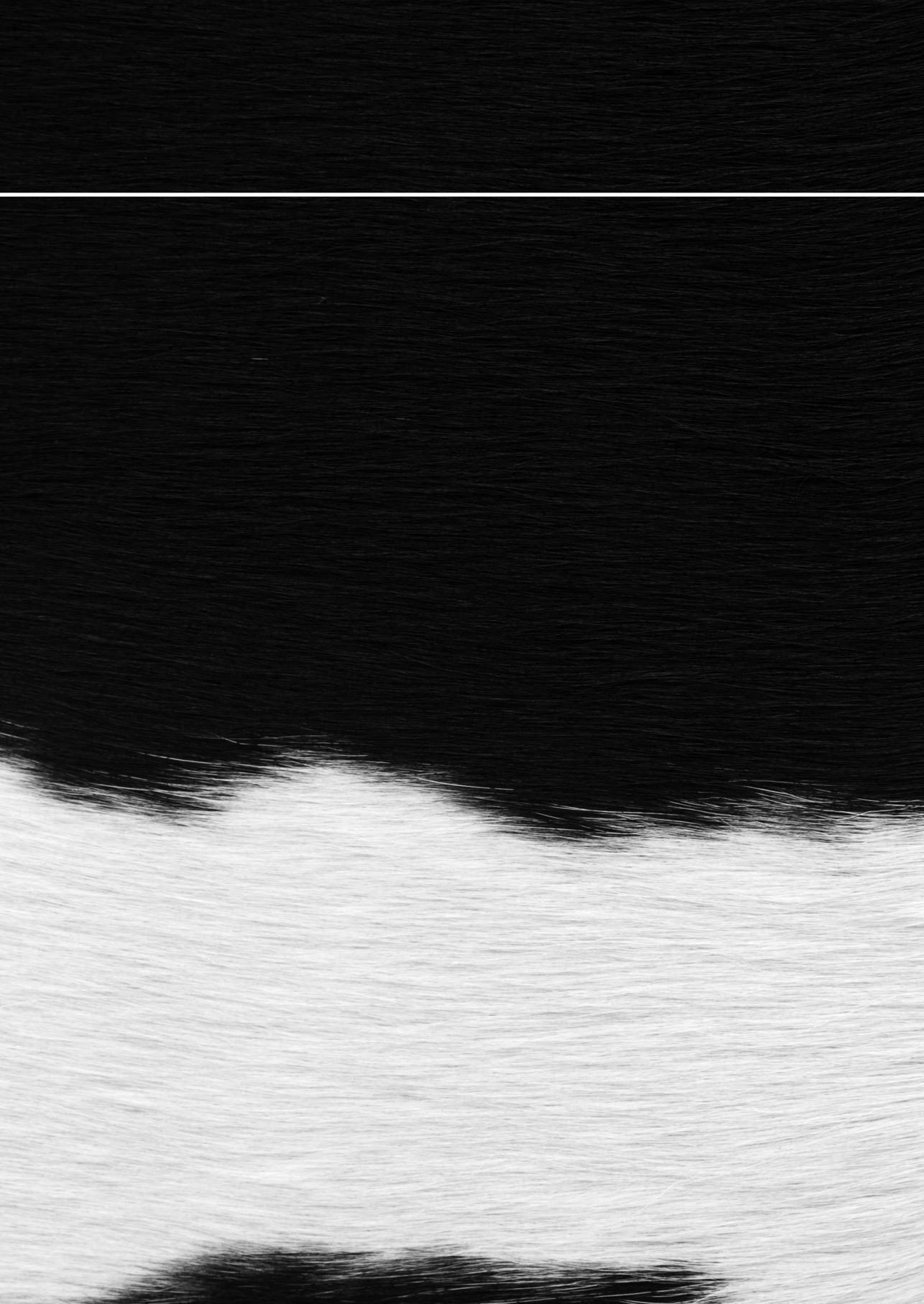

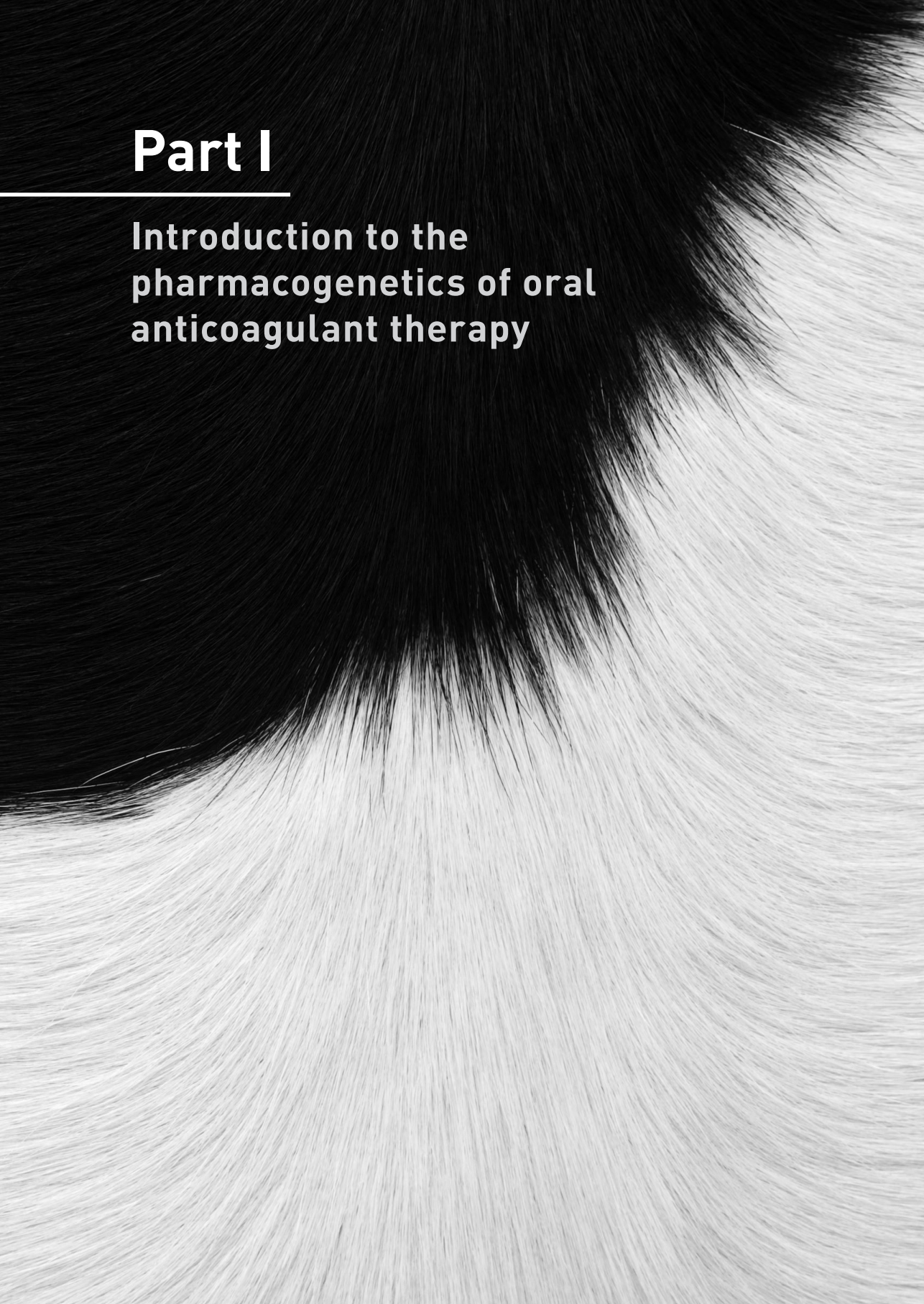

Part I

Introduction to the pharmacogenetics of oral anticoagulant therapy

Chapter 2

Pharmacogenetics of oral anticoagulant therapy

Rianne M.F. van Schie, Talitha I. Verhoef, Anthonius de Boer, Felix J.M. van der Meer, Tom Schalekamp, and Anke-Hilse Maitland-van der Zee

Abstract

Coumarins are effective drugs for treatment and prevention of thromboembolic events. However, patients are balancing between underdosing (which increases the risk of thromboembolic events) and overdosing (which increases the risk of hemorrhages). It has been shown that polymorphisms in *VKORC1* and *CYP2C9* explain a large part (35-50%) of the dose variability. Also patient characteristics and environmental factors play a role. Currently, clinical trials are performed to investigate the added value and cost effectiveness of pretreatment genotyping. In this chapter, the pharmacogenetics of oral anticoagulant therapy will be discussed, including ongoing clinical trials and the cost-effectiveness.

Introduction

Coumarin derivatives, such as warfarin, phenprocoumon and acenocoumarol, are very effective in the prevention and treatment of thromboembolic diseases, for example in patients with atrial fibrillation or venous thromboembolism[1-5]. Patients with atrial fibrillation have an annual stroke risk of 4.5% and during treatment with warfarin, this risk decreases to 1.4%[1]. Warfarin is the most prescribed coumarin in the world while phenprocoumon and acenocoumarol are the coumarins of first choice in continental Europe[6-8]. These drugs are already on the market for decades, but finding the right dose for each patient is still challenging. Coumarins have a small therapeutic index, often resulting in a too low anticoagulant effect with an increased risk of thromboembolism or a too high anticoagulant effect with an increased risk of hemorrhages[9-13]. Furthermore, they are subject to inter- and intra-individual variability in dose requirements[14, 15]. Also, the use of coumarins frequently results in drug-related hospitalization[16-19]. It has been established that anticoagulation response is affected by environmental, clinical, and genetic factors such as age, height, weight, concurrent drug therapy, morbidities, dietary vitamin K intake, and genetic variation in Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase complex subunit 1 (VKORC1)[20-25]. This chapter will elaborate on the inter- and intra-patient variability of coumarins with the focus on the pharmacogenetics of the anticoagulant therapy.

Mechanism of action

Inactive coagulation factors II, VII, IX and X require γ-carboxylation of the glutamic acid (Glu) residues into γ-carboxyglutamic (Gla) residues for their coagulation activity[26-28]. In this process, the γ-carboxylase cofactor vitamin K-hydroquinone is oxidized to vitamin K-epoxide. Vitamin K- epoxide is recycled for the carboxylation of new coagulation factors in a 2-step reduction to vitamin K-hydroquinone[27, 28]. Vitamin K epoxide reductase (VKOR) is the catalyzer of the first step in the reduction of vitamin K-epoxide into vitamin K-quinone and also contributes to the second reduction step, in which vitamin K-quinone is further reduced to vitamin K-hydroquinone[27, 28]. Cytochrome P450 4F2 (CYP4F2) is a vitamin K-oxidase and metabolizes vitamin K-quinone to hydroxyvitamin K[29]. Coumarins, also called vitamin K antagonists, inhibit the reduction of oxidized vitamin K by binding to a small trans membrane protein in the endoplasmatic reticulum called vitamin K epoxide reductase complex subunit 1 (VKORC1), which is part of the VKOR complex[30, 31]. As a result, vitamin K-hydroquinone will not become available for the γ-carboxylation of coagulation factors (see Figure 1). Coumarins thus act indirectly on the coagulation factors. The

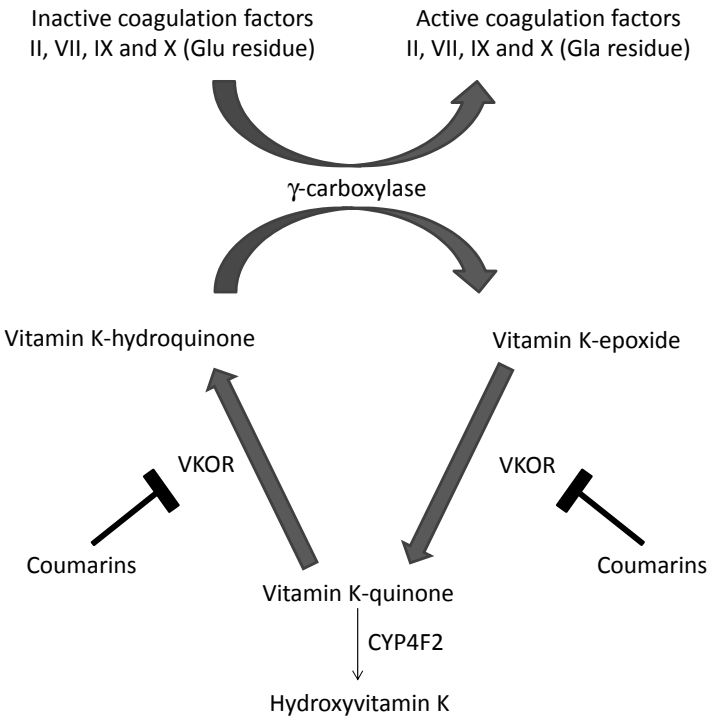

Figure 1. The mechanism of action[26-28].

half-lives of the coagulation factors ranges from approximately 6 hours for factor VII to 2.5 days for factor II (prothrombin)[32]. This means that the effect of the coumarins in inducing an anticoagulant effect starts 15 hours after administration[33] and the effect is complete after 36 to 72 hours after start of coumarin use[34, 35].

Pharmacokinetics

Even though the mechanism of action is identical for the three coumarins, there are clear differences in their pharmacokinetic properties. We will therefore discuss the pharmacokinetics of the coumarins separately.

Warfarin

Warfarin is metabolized to five different monohydroxylated metabolites (i.e. 4'-, 6-, 7-, 8- and 10-hydroxywarfarin), cis- and trans-dehydro-warfarin, and two diastereomeric alcohols[36, 37]. Metabolism to hydroxylated and dehydro- metabolites is dependent on Cytochrome P450 (CYP) enzymes and occurs in the microsomal fraction of hepatocytes[38], while reduction to alcohols is dependent on NADPH and takes place in

the endoplasmatic reticulum and cytosol[39, 40]. Different monohydroxylated warfarin metabolites are formed, which suggests involvement of different CYP-isoenzymes. The largest proportion of hydroxylation is catalyzed by CYP2C9, resulting in the formation of 7-hydroxywarfarin, the most abundant metabolite. To a much smaller extent, CYP2C8, CYP2C19, CYP1A2 and CYP3A4 are involved[37]. The half-life of warfarin is 24-33 hours for S-warfarin and 35-58 hours for R-warfarin[37, 41].

Acenocoumarol

Acenocoumarol is metabolized to 6-, 7-, and 8-hydroxy-acenocoumarol, amino and acetamido acenocoumarol and two diastereometic alcohols[42, 43]. Enzymes involved in the formation of amino and acetamido metabolites and alcohols have not yet been identified. Hydroxylation is dependent on CYP-enzymes[44]. Hydroxylation is catalyzed by CYP2C9, the main metabolite being 7-hydroxyacenocoumarol. As for warfarin, CYP2C9 regioselectivity for the 6- and 7- position and stereoselectivity for the S-enantiomer seem to play a role[37].To a much smaller extent, CYP2C19 and CYP1A2 are involved[37]. The half-life of acenocoumarol is 1.8 hours for S-acenocoumarol – the most potent form- and 6.6 hours for R-acenocoumarol[43].

Phenprocoumon

The metabolites of phenprocoumon are 4'-, 6-, 7- and 8-hydroxy-phenprocoumon and in contrast to warfarin and acenocoumarol all metabolites are hydroxyl-metabolites[37]. The hydroxyl-metabolites are all formed by CYP-enzymes[45, 46]. The 6- and 7-hydroxy phenprocoumon are the most abundant metabolites, 45% and 52% respectively[37]. The main metabolizing enzymes involved in the formation of these metabolites are CYP2C9 (approximately 60-65%) and CYP3A4 (approximately 35-40%). These CYP-enzymes and CYP2C8 are also involved in the formation of the other metabolites[37]. The half-life of phenprocoumon is much longer compared with the two other coumarins; 110-130 hours for S-phenprocoumon – the most potent form- and 110-125 hours for R-phenprocoumon[47].

Anticoagulant therapy

In order to find the most effective and safe balance between undercoagulation with a risk of thromboembolic events and overcoagulation with a risk of hemorrhage, it was recommended during the first American College of Chest Physicians (ACCP) conference that therapy with coumarins should be monitored by the International Normalized Ratio (INR) as established by the World Health Organization[48, 49]. A dose that prolongs the INR to two to three times control (i.e. INR of 2.0 to 3.0) was recom-

mended for indications such as prophylaxis and treatment of venous thromboembolism, and atrial fibrillation[49]. Higher ranges (i.e. INR of 3.0 to 4.5) were recommended for patients with artificial heart valves and recurrent venous thrombosis despite adequate anticoagulation[49]. These recommendations are widely accepted and increased the safety of coumarins[48]. In the Netherlands, there are specialized anticoagulation clinics that follow dosing strategies to maintain the INR between the 2.0 and 3.5 for the low intensity range (e.g. atrial fibrillation, venous thromboembolism) or 2.5 and 4.0 for the high intensity range (e.g. artificial heart valves, recurrent venous thrombosis despite adequate anticoagulation)[28, 50, 51]. Dutch patients regularly visit the anticoagulation clinic for INR measurements and subsequent dose adjustments. Anticoagulation clinics improve the quality of the anticoagulant therapy and are cost saving because hemorrhages and thromboembolic events are prevented more adequately if compared to usual clinical care[52, 53]. The Dutch anticoagulation clinics achieved a median percentage time spend in target INR range of 77.9% for patients in the low intensity range and 73.2% for patients in the high intensity range in 2010[50]. This is a very high percentage time in range compared with other countries (for example, 63% in the UK, 56% in Germany, and 66% in Austria) and comparable to Sweden (76%)[54], but it still means that over 20% of the time, INRs are above or below the target range. This can be explained by intra-individual dose variability over time, which will be discussed, together with inter-individual variability, in the next paragraph.

Inter- and intra-individual dose variability

The coumarin dose that is optimal for one patient, may cause hemorrhages in another patient and thromboembolic events in a third patient. Patients need very different dosages which can differ up to 10 fold[14]. For example, the maintenance dose of warfarin ranges from 1.5 to 12 mg/day, acenocoumarol from 1 to 9 mg/day and phenprocoumon from 0.75 to 9 mg/day[37]. In addition, the required dose may also change over time in an individual patient. There are several factors that cause inter- and intra-individual variability.

Patient characteristics and environmental factors

Effects of patient characteristics and environmental factors can roughly be divided into 3 categories: effects on the coumarin dose, effects on the stability of the anticoagulant therapy, and effects on clinical outcomes (e.g hemorrhages and thromboembolic events).

Effects on coumarin dose

With increasing age, the coumarin dose requirements decrease, and coumarin dosages increase with increasing weight and height[25, 55]. Many diseases affect the coumarin dosages as well. Patients with hepatic disorders need lower dosages because the synthesis of coagulation factors is reduced in these patients[56, 57]. Hyperthyroidism leads to decreased coumarin dosages, while hypothyroidism is associated with a decreased catabolism of vitamin K-dependent coagulation factors, attenuating the response to oral anticoagulant therapy and resulting in increased dose requirements[56]. Heart failure may cause hepatic congestion resulting in a decreased synthesis of coagulation factors and therefore lower coumarin maintenance dose requirements[56, 58]. Malignancies might affect the coumarin dose by metastatic liver disease, malnutrition, or use of chemotherapy[56]. Fever decreases coumarin dose requirements probably by increasing degradation of coagulation factors[9]. Dehydration might affect the INR and therefore the coumarin dose by changing the volume of distribution of the coumarins[57]. Hypo-albuminuria affects the concentration of unbound coumarins and therefore the coumarin dose requirements[57]. Kidney disorders might also affect the albumin concentration and therefore coumarin dose requirements[57]. Comedication use is also of importance. There are many drugs that influence the coumarin dose requirements. Dependent on the drug, the anticoagulation effect is increased or decreased[22, 23, 25, 59-62]. In the Netherlands, clinically relevant drug interactions with coumarins have been described and regulated in the 'Standard management coumarin interactions'[63, 64]. There are two main categories of drug interactions: first, the pharmacokinetic interactions affecting the absorption, distribution or elimination and second, the pharmacodynamic interactions affecting production or metabolism of coagulation factors, or directly affecting coagulation[57]. Besides affecting the coumarin maintenance dose, comedication might also increase the risk of hemorrhages.

Effects on stability of the anticoagulant therapy

Dietary vitamin K intake interferes with the stability of the oral anticoagulant therapy[66]. Daily supplementation of vitamin K intake contributes to a more stable anticoagulant therapy[67-69]. Also other nutrition factors can be of influence[57]. Because vitamin K is a fat-soluble vitamin, the resorption of vitamin K through the intestines is influenced by fat intake and resorption disorders which might result in instability of the anticoagulant therapy. Gavage feeding might cause fluctuating INRs[57, 70] due to different concentrations of vitamin K in the gavage in comparison to normal diet, vitamin K might bind to proteins in the gavage feeding, and in addition, vitamin K might get lost in the preparation of the gavage or due to adsorption to the tube wall. Disorders of the gastrointestinal tract (e.g. vomiting, diarrhea, malabsorption of fat, or antibiotic use which may affect bacteria in the intestines that produce

vitamin K) might affect the stability of anticoagulant therapy[57]. Increased levels of stress are thought to be associated with increased INRs and varying amounts of physical exercise may cause a fluctuation in INR as well[57]. Travelling, and thus possibly a change in diet, a lowered compliance, and increased alcohol consumption, might cause instability as well[57]. Poor compliance also contributes to instability of patients, but it is only a minor factor[65].

Effects on clinical outcomes
Hematological disorders might affect the anticoagulant therapy by increasing the risk of hemorrhage; for example local disorders such as polyps increase the risk of hemorrhage. Malignancies may both increase the risk of venous thromboembolism and hemorrhages[57].

Pharmacogenetics

In 1992, Rettie *et al*. reported that CYP2C9 is the main metabolizing enzyme of warfarin[71]. Also CYP1A2 and CYP3A4 contribute to the metabolism of the drug[71]. Furuya *et al*. hypothesized that polymorphisms in *CYP2C9* (resulting in proteins with different catalytic activities) might have a major effect on the clearance of the most potent enantiomer (S-warfarin) and therefore might affect the warfarin maintenance dose[72]. They recruited almost 100 patients that attended the anticoagulation clinic for routine INR monitoring. Information on body weight, height, age, sex, drug history, INRs history, indication for coumarin use, and comorbidities was collected. A blood sample was used to determine the *CYP2C9*2* genotype. Of the 94 included patients, 58 (62%) were wild type (*CYP2C9*1/*1*) and 36 (38%) heterozygous for *CYP2C9*2*. There were no patients homozygous for *CYP2C9*2*. Patients carrying the variant allele required significantly lower warfarin dosages than wild type patients (Mann-Whitney U-test, p=0.02). In addition, they found an association between age and warfarin dose requirements. The results suggesting an effect of *CYP2C9* genotypes on the coumarin maintenance dose have been replicated by many research groups[25, 73-78]. Not only *CYP2C9*2*, but also *CYP2C9*3* is a common variant allele in Caucasians that reduces the coumarin maintenance dose significantly[25, 73-78]. The *CYP2C9*2* allele frequencies vary from 8 to 19% and the *CYP2C9*3* alleles from 3 to 16% in Caucasians[79]. In East-Asian and African or Afro-American populations, *CYP2C9*2* is absent and *CYP2C9*3* has lower allele frequencies[79]. The *CYP2C9* genotype explains approximately 4.5-17.5% of the coumarin (warfarin, acenocoumarol and phenprocoumon) dose variation[25, 76, 80-85].

Rost *et al*. and Li *et al*. identified VKORC1 as target of the coumarins in 2004[30, 31]. This introduced a new possibility for explaining the coumarin dose variability. And indeed, many researchers showed decreased coumarin dose requirements if patients carried one or two variant alleles in the *VKORC1* gene[73-75, 82, 86, 87]. Two SNPs in *VKORC1*, the -1639G>A and the 1173 C>T, were associated with decreased warfarin dose requirements[28]. It was demonstrated that promotor SNP -1639G>A causes the variability in VKORC1 activity by suppressing the gene expression, but a role for 1173 C>T could not be excluded because of the complete linkage disequilibrium between the two SNPs[88]. Patients carrying one or two variant alleles have decreased levels of *VKORC1* mRNA in the liver and therefore need lower coumarin dosages if compared to wild type patients[88]. Because the two SNPs are in complete linkage disequilibrium[88, 89], studying either of the two SNPs will give the same results. Allele frequencies for the *VKORC1* variant allele are 37-41% in Caucasians, 10-12% in African Americans, and 88-92% in East-Asians[28].

There are many other genes that could potentially affect the coumarin maintenance dose. The association with the coumarin dose might for example be based on other pharmacokinetic or pharmacodynamic mechanisms, for example by affecting transport of coumarins or vitamin K or, by affecting the vitamin K cycle. In the metabolism of phenprocoumon, other metabolizing enzymes, especially CYP3A4, also play an important role[37, 90] and therefore SNPs in the genes encoding for these metabolizing enzymes are hypothesized to affect the phenprocoumon dose requirements. However, Teichert *et al.* did not find an association between *CYP3A4*1B* and the phenprocoumon dose[91]. Another gene that has been associated with coumarin response is *CYP4F2*[91-97]. CYP4F2 is a vitamin K oxidase. Patients carrying one or two V433M variant alleles in *CYP4F2* have a reduced capacity to metabolize vitamin K, resulting in increased vitamin K levels and therefore also resulting in higher coumarin dose requirements if compared to non-carriers[29]. SNPs in *CYP4F2* have a nominal effect on the coumarin maintenance dose; it explains an additional 1 to 2% of the coumarin dose requirements[92, 94]. Polymorphisms in *GGCX*, the gene encoding γ-glutamylcarboxylase, which is involved in the carboxylation of coagulation factors also have been shown to have a minor effect on the coumarin dose[74, 98] however other research groups did not find an association between the coumarin dose and polymorphisms in *GGCX*[99, 100]. Other minor influences on the coumarin maintenance dose might be caused by polymorphisms in the genes encoding for the coagulation factors VII and X[101], *EPHX1*[100, 102] which encodes a protein subunit of VKOR, and *APOE*[103-107] which encodes for the protein responsible for the vitamin K uptake, and in *PROC*[103] which encodes for protein C, responsible for the inactivation of coagulation factors Va and VIIIa. All these polymorphisms show low or no clinical relevance.

Until now, only *VKORC1*, *CYP2C9* and *CYP4F2* genotypes were found to be associated with the coumarin maintenance dose in genome wide association studies (GWAS)[91, 93, 94, 97]. One study also found an association with *CYP2C18* and the acenocoumarol dose[97]. In addition, a study performed in 1496 Swedish patients starting warfarin treatment investigated the impact of 183 polymorphisms in 29 candidate genes for an association with the warfarin dose[83].They only found an association for *CYP2C9* and *VKORC1*.

C*YP2C9* and *VKORC1* genotypes together explain approximately 35-50% of the coumarin dose requirements[83, 87, 108]. To date, a number of studies have reported the development of pharmacogenetics-guided algorithms for coumarins in order to predict the personalized coumarin dose before start of the anticoagulant therapy[25, 76, 80-85], of which one is developed in the context of this thesis for the EU-PACT trial (Chapter 3).

Clinical trials

Currently, several RCTs are ongoing testing the added value of pretreatment genotyping[109-111], which means that the *VKORC1* and *CYP2C9* genotype of the patient are determined and the starting dose and maintenance dose are estimated using an algorithm in which these genotypes are being used compared to a group in which these genotypes are not used. This thesis is based on the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial (Chapter 5) (unique ClinicalTrials.gov Identifiers: NCT01119274, NCT01119261, and NCT01119300)[109]. It is the only RCT that investigates three coumarins (warfarin, phenprocoumon and acenocoumarol). The EU-PACT trial compares a dose algorithm with patient characteristics, e.g. age, height, weight, sex, and amiodarone use (or in the case of warfarin standard clinical care), to a dose algorithm including the aforementioned patient characteristics, and *VKORC1* and *CYP2C9* genotype. The primary outcome is the time within target INR range.

Other RCTs currently recruiting patients are COAG (NCT00839657)[110] and GIFT (NCT01006733)[111]. CoumaGen-II (NCT00927862) already completed the study in June 2011 and recently published their results[112]. They showed that pharmacogenetic dosing was superior to standard dosing for percentage time in and out of therapeutic range.

When the results of clinical trials investigating the effect of genotyping before starting the use of coumarins become available, a decision should be made whether to

implement genotype-guided dosing or not. This decision will not only depend on the effectiveness of genotyping, but also on the cost-effectiveness since an important factor for implementation will be reimbursement of the genetic test by health insurance companies.

Conclusion

Coumarins are effective drugs for treatment and prevention of thromboembolic events. However, patients are balancing between underdosing (which increases the risk of thromboembolic events) and overdosing (which increases the risk of hemorrhages). It has been shown that polymorphisms in *VKORC1* and *CYP2C9* explain a large part (35-50%) of the dose variability. Also patient characteristics and environmental factors play a role. Currently, clinical trials are performed to investigate the added value and cost effectiveness of pretreatment genotyping.

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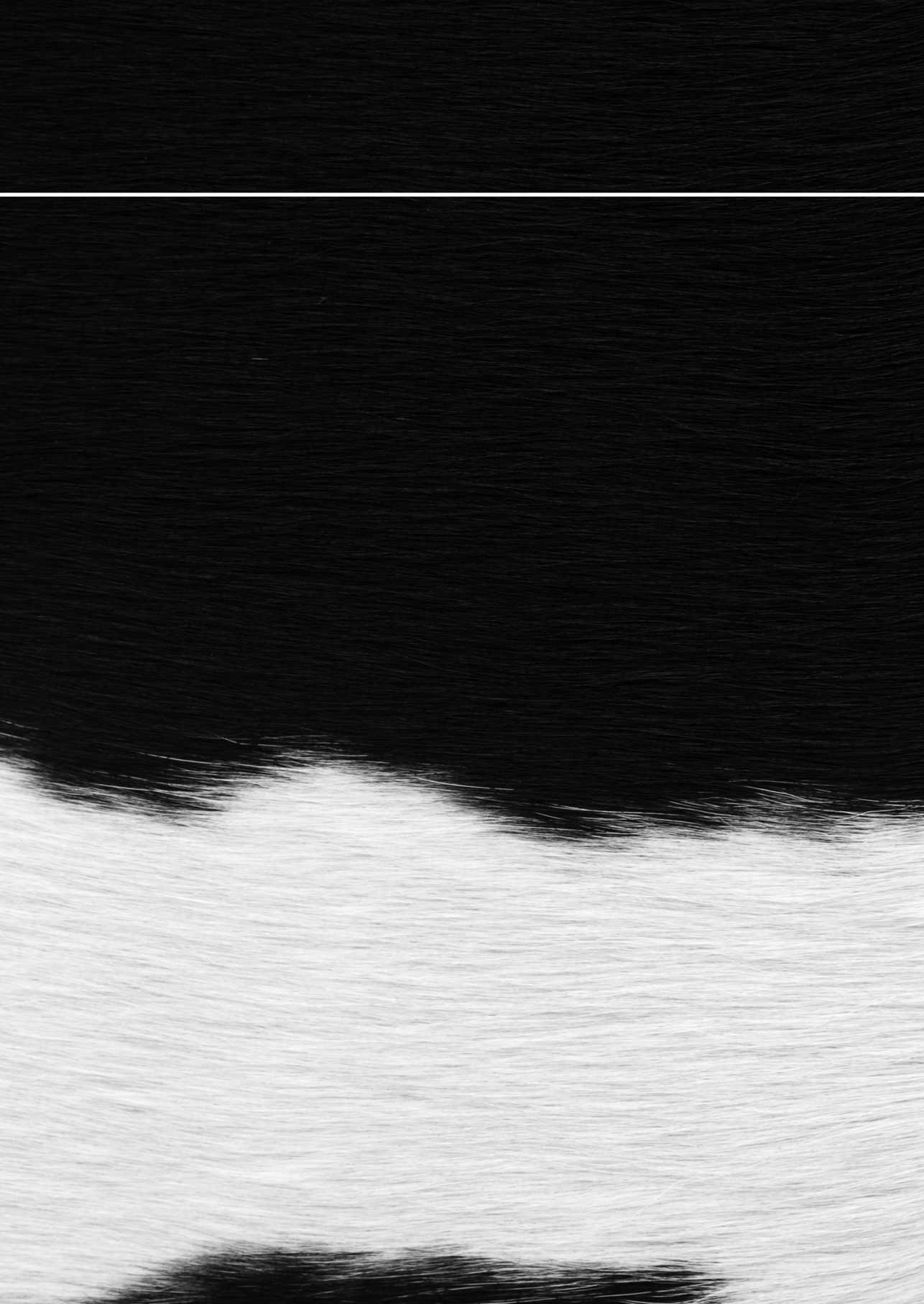

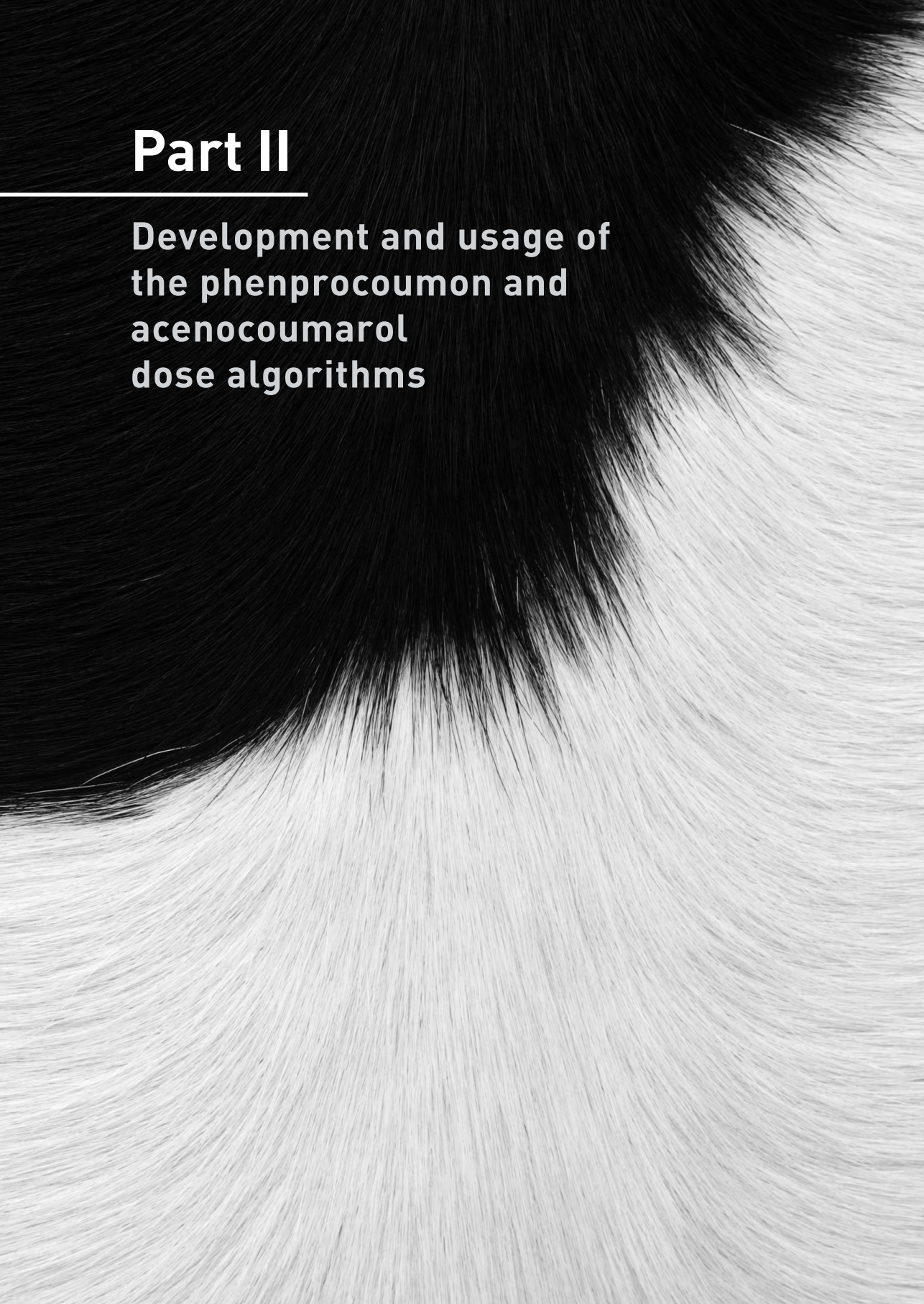

Part II

Development and usage of the phenprocoumon and acenocoumarol dose algorithms

Chapter 3

Loading and maintenance dose algorithms for phenprocoumon and acenocoumarol using patient characteristics and pharmacogenetic data

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Abstract

Aim

Polymorphisms in *CYP2C9* and *VKORC1* influence patients' phenprocoumon and acenocoumarol dose requirements. To provide physicians with tools to estimate the patient's individual dose, we aimed to develop algorithms for phenprocoumon and acenocoumarol.

Patients & methods

In two Dutch anticoagulation clinics, data on age, sex, height, weight, comedication, coumarin derivative doses, and international normalized ratio values were obtained from 624 patients taking phenprocoumon and 471 taking acenocoumarol. Single Nucleotide Polymorphisms relevant to coumarin derivative dosing on the *CYP2C9* and *VKORC1* genes were determined. Using multiple linear regression, we developed genotype-guided and nongenotype-guided algorithms to predict the maintenance dose with patient characteristics and genetic information. In addition, loading doses were derived from the calculated maintenance doses.

Results

We performed external validation in an independent data set with 229 phenprocoumon and 168 acenocoumarol users. *CYP2C9* and *VKORC1* genotype, weight, height, sex, age, and amiodarone use contributed to the maintenance dose of phenprocoumon and acenocoumarol. The genotype-guided algorithms explained 55.9% (phenprocoumon) and 52.6% (acenocoumarol) of the variance of the maintenance dose, the non-genetic algorithms 17.3% (phenprocoumon) and 23.7% (acenocoumarol). Validation in an independent data set resulted in an explained variation of 59.4% (phenprocoumon) and 49.0% (acenocoumarol) for the genotype-guided algorithms and for 23.5% (phenprocoumon) and 17.8% (acenocoumarol) for the nongenotype-guided algorithms, without height and weight as parameters.

Conclusion

To our knowledge, these are the first genotype-guided loading and maintenance dose algorithms for phenprocoumon and acenocoumarol using large cohorts. The utility of these algorithms will be tested in randomized controlled trials.

Introduction

Patients receiving coumarin therapy are at risk of thrombosis and therapy failure due to underdosing or at risk of hemorrhage due to overdosing[1,2], making coumarins often associated with drug-related hospitalization[3-5]. This is because coumarins have a narrow therapeutic window and there is wide inter- and intra-individual variability in dose requirements[6,7]. Therefore, patients are monitored by measuring the International Normalized Ratio (INR). Current clinical practice is that all patients receive a standard loading dose at the start of the therapy, which is subsequently adjusted to an individual maintenance dose according to the measured INR. This leads to a mean percentage time within the target INR ranging from only 45 to 64% during the first 2 months of the anticoagulant therapy[8-10], which needs to be improved.

Developing a strategy towards more individualized dosing of coumarins has gained interest in recent years. It is known that patient characteristics such as age and body size influence the dose requirements. More recently, genetic factors, notably polymorphisms in the *VKORC1* gene which expresses vitamin K epoxide reductase (the main target for coumarins) and the *CYP2C9* gene which expresses cytochrome P450 2C9 (the enzyme responsible for the metabolism of coumarin), together have been shown to explain 35–50% of the inter-individual variability in dose requirements[11]. To date, a number of studies have reported the development of pharmacogenetics-guided algorithms for warfarin[12-18]. However, there are no published reports on the development and validation of algorithms in a large cohort for predicting the loading and maintenance dose of phenprocoumon and acenocoumarol. In continental Europe, phenprocoumon and acenocoumarol are most commonly used for anticoagulant therapy; for example in 2008 in the Netherlands, >200 000 prescriptions were made for phenprocoumon and >1 million were made for acenocoumarol[19]. There is a definite need for the development of more refined algorithms for both these drugs if the notion of a future approach to individualized therapy is to be realized. The aim of this study was to derive algorithms to estimate the individualized loading and maintenance doses for phenprocoumon and acenocoumarol before the start of the treatment.

Patients & methods

Study design & patients

Patients currently using either phenprocoumon or acenocoumarol were eligible to take part in the study if aged 18 years and over and with a target INR in the lowest

intensity category (according to Dutch guidelines INR 2.0–3.5). Pregnant or breast-feeding women, patients who were in a nursing home, and patients participating in other clinical studies were excluded. Eligible patients who had a scheduled visit at the anticoagulation clinic from either 10 to 12 November 2009 (Anticoagulation Clinic Leiden, phenprocoumon) or from 23 to 27 November 2009 (Anticoagulation Clinic Medial, acenocoumarol) were invited to participate. We aimed to include approximately 1000 patients because that would increase the probability of capturing data on a reasonable number of patients (at least five patients per coumarin group) having the least frequent *CYP2C9* genotypes (e.g. *CYP2C9*2/*3* and **3/*3*) and therefore to assure accurate dose estimates for all genotypes. The Committee Medical Ethics Leiden approved the study protocol, and patients provided informed consent before inclusion into the study.

Data collection & genotyping

Height, current weight (weight at the moment of inclusion), and weight at the start of the anticoagulant therapy were recorded for each participant. Data on the participants' age, sex, history of comedication, history of INR values, and prescribed coumarin doses were obtained from the electronic registry databases of the anticoagulation clinics. Since 1983, in the Netherlands at each visit to the anticoagulation clinic, INR measurements, prescribed doses, and comedication are routinely collected and recorded in registry databases. Residual blood samples from INR measurements were used to genotype the patient for *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), and *VKORC1 1173C>T* (rs9934438) using predesigned Taqman assays (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and according to the manufacturers' protocol. *CYP2C9*1* and *VKORC1* C genotypes were assigned if polymorphisms in the analysed corresponding Single Nucleotide Polymorphisms (SNPs) (*CYP2C9*2*, *CYP2C9*3*, and *VKORC1 1173C>T*) were lacking. Other variant alleles are rare in Caucasians. Therefore, there is a negligible risk for a misclassification of phenotypes due to other variant alleles. Genotypes were determined on LightCycler® 480 (Roche Diagnostics, Almere, the Netherlands) in 384-well plates that include positive (previous established genotype) and negative controls (Tris-EDTA buffer). In addition, as quality control 10% of the samples were genotyped in duplicate.

Outcome & determinants

The mean stable coumarin maintenance dose in mg/day at the first stable period after initiation of anticoagulant therapy was used as the outcome measure. A stable period was defined as a period of at least 3 weeks with three or more consecutive INR measurements within target range with <10% change in the coumarin dose. To develop the nongenotype-guided algorithms, the *a priori* defined determinants

were age in years, sex, amiodarone use, height in centimetres, and weight in kilograms at the start of the anticoagulation treatment (if missing, current weight was used instead). For the genotype-guided algorithm, *CYP2C9* and *VKORC1* genotypes were used as additional determinants.

Statistical analysis & algorithm development

Multiple linear regression was used to estimate the maintenance dose of phenprocoumon and acenocoumarol. To reduce the influence of extreme observations, the values of continuous predictive variables were truncated at approximately the 2.5th and 97.5th percentile[20]. Either patients with missing values for at least one of the determinants or those who did not reach a stable phase within a year following the start of the therapy were excluded. The optimal transformation of the outcome (original scale, log transformation, or square root transformation) was determined by selecting the transformation with the average lowest mean squared error (which is the mean of the square of the difference between observed and predicted outcome) and the average lowest mean absolute error (which is the mean of the absolute difference between predicted and observed coumarin doses). The interactions between the determinants were evaluated for any possible improvement in the algorithms. The algorithms were internally validated by calculating the coefficient of determination (R^2) and the mean absolute error.

The algorithms were externally validated using two data sets of Schalekamp *et al*.[21,22] These data sets contain complete data of 229 patients using phenprocoumon and 168 patients using acenocoumarol. Because weight and height were not available in the validation data sets, we validated the algorithms without height and weight. In addition, we validated the algorithms using multiple imputation methods for missing weight and height (see Supplementary material online). The R^2 between the algorithm predictions and the observed outcomes, the mean squared error and the mean absolute error were calculated.

The loading dosages were derived from the estimated maintenance dose. In general, only drugs with a long elimination half-life are candidates for loading dose administration at the start of the therapy for rapid achievement of the steady-state plasma drug concentration and therapeutic effect[23]. Acenocoumarol has a relatively short half-life of 8-14 h and therefore loading doses are not necessary. Therefore, our recommended acenocoumarol loading doses are rounded values of the calculated individual maintenance doses. Phenprocoumon on the other hand has an average half-life of 160 h and therefore with loading doses a faster therapeutic response is attained[24,25].

In general, the loading dose can be calculated from the maintenance dose by using a first-order kinetics equation[23]. The current standard clinical practice for phenprocoumon is to divide the loading dose over the first 3 days of therapy. The loading dose is obtained from the calculated stable maintenance dose using the equation (1)

$$MD = \frac{D_1 \cdot e^{-2\kappa} + D_2 \cdot e^{-\kappa} + D_3}{1 - e^{-\kappa}} \quad (1)$$

with MD defined as the maintenance dose, D_1 the dose received on day 1, D_2 the dose received on day 2, and D_3 the dose received on day 3, $\kappa=\ln(2)/t_{1/2}$, where κ is the elimination rate constant and $t_{1/2}$ is the drug half-life in days.

Anticoagulation response to coumarins is the result of a complex interplay between several variables, including vitamin K availability and the presence of functional vitamin K-dependent clotting factors. However, inhibition of the synthesis of functional vitamin K-dependent clotting factors II, VII, IX, and X is dependent on the plasma coumarin concentration, which in turn is related to the CYP2C9 enzyme activity. Therefore, on this basis equation (1) above was used for estimating phenprocoumon loading dose which to some extent reflects inter-patient CYP2C9 variability.

A number of restrictions were applied for the phenprocoumon loading dose estimation to minimize the risk of over- or underdosing, especially for the nongenotype-guided algorithm (see Supplementary material online). We used the statistical software SPSS (PASW Statistics) version 18 for the analysis.

Results

Patient cohort

In total, 624 patients using phenprocoumon and 471 patients using acenocoumarol were included in the study. For the nongenotype-guided algorithm, complete data were available for 587 patients using phenprocoumon and 400 patients using acenocoumarol. For the genotype-guided algorithm, data on 559 phenprocoumon and 375 acenocoumarol patients were available; see flowcharts Figure 1. The median maintenance dose for phenprocoumon was 2.12 mg/day and for acenocoumarol was 2.34 mg/day. Patient characteristics are presented in Table 1. Height, weight, age, and *CYP2C9* and *VKORC1* genotype were not significantly different among the group with and group without missing values, except for *VKORC1* genotype distribution in the acenocoumarol patients (p=0.037).

Genotyping

No inconsistencies were observed for the quality controls. Allele frequencies for *CYP2C9* were 0.82 for the wild-type allele, 0.12 for *CYP2C9*2*, and 0.07 for *CYP2C9*3*. Allele frequencies for *VKORC1 1173C>T* were 0.61 for C and 0.39 for T. All three genotype distributions followed Hardy–Weinberg equilibrium.

Phenprocoumon & acenocoumarol maintenance dose algorithm

The square root of the maintenance dose in mg/day during the first stable INR monitoring period was on average the best outcome transformation for the four algorithms and was therefore chosen as the outcome measure. Differences between this and other transformations were very small (see Supplementary material online).

The intercept and coefficients of all four algorithms as well as the univariate R^2 for the parameters included are presented in Table 2. There were no interactions between the determinants that improved the algorithms.

The explained variance was 55.9% (phenprocoumon) and 52.6% (acenocoumarol) for the genotype-guided algorithms and 17.3% (phenprocoumon) and 23.7% (acenocoumarol) for the nongenotype-guided algorithms. The mean absolute error for the genotype-guided algorithms was 0.45 mg/day (phenprocoumon) and 0.52 mg/day (acenocoumarol) and for the nongenotype-guided algorithms 0.63 mg/day (phenprocoumon) and 0.70 mg/day (acenocoumarol).

Phenprocoumon & acenocoumarol loading dose strategies

Table 3 shows the loading dose corresponding to a given maintenance dose for phenprocoumon. A dosing regimen of 9 mg on day 1, 6 mg on day 2, and 6 mg on day 3 as a loading schedule (or alternatively 12-6-3 mg for the first 3 days) is currently the maximum standard loading dose for phenprocoumon. On the basis of our data, we recommend a higher loading dose (9, 9, and 6 mg on days 1, 2, and 3, respectively) compared with the current standard regimen only when it is 90% certain that the patient needs a higher loading dose than standard clinical care. This is valid for patients who according to the genotype-guided algorithm have a predicted maintenance dose of 2.92 mg/day or higher. In addition, 3, 3, and 3 mg as a loading schedule on days 1, 2 and 3 (corresponding to a maintenance dose of <1.04 mg/day) is only been given to patients who are dosed according to the genotype-guided algorithm, since such low doses will not be calculated with the nongenotype-guided algorithm. Figure 2 shows the distribution of the loading dose regimens using the genotype-guided algorithm and the nongenotype-guided algorithm in the derivation cohort. Table 4 shows the loading dose corresponding

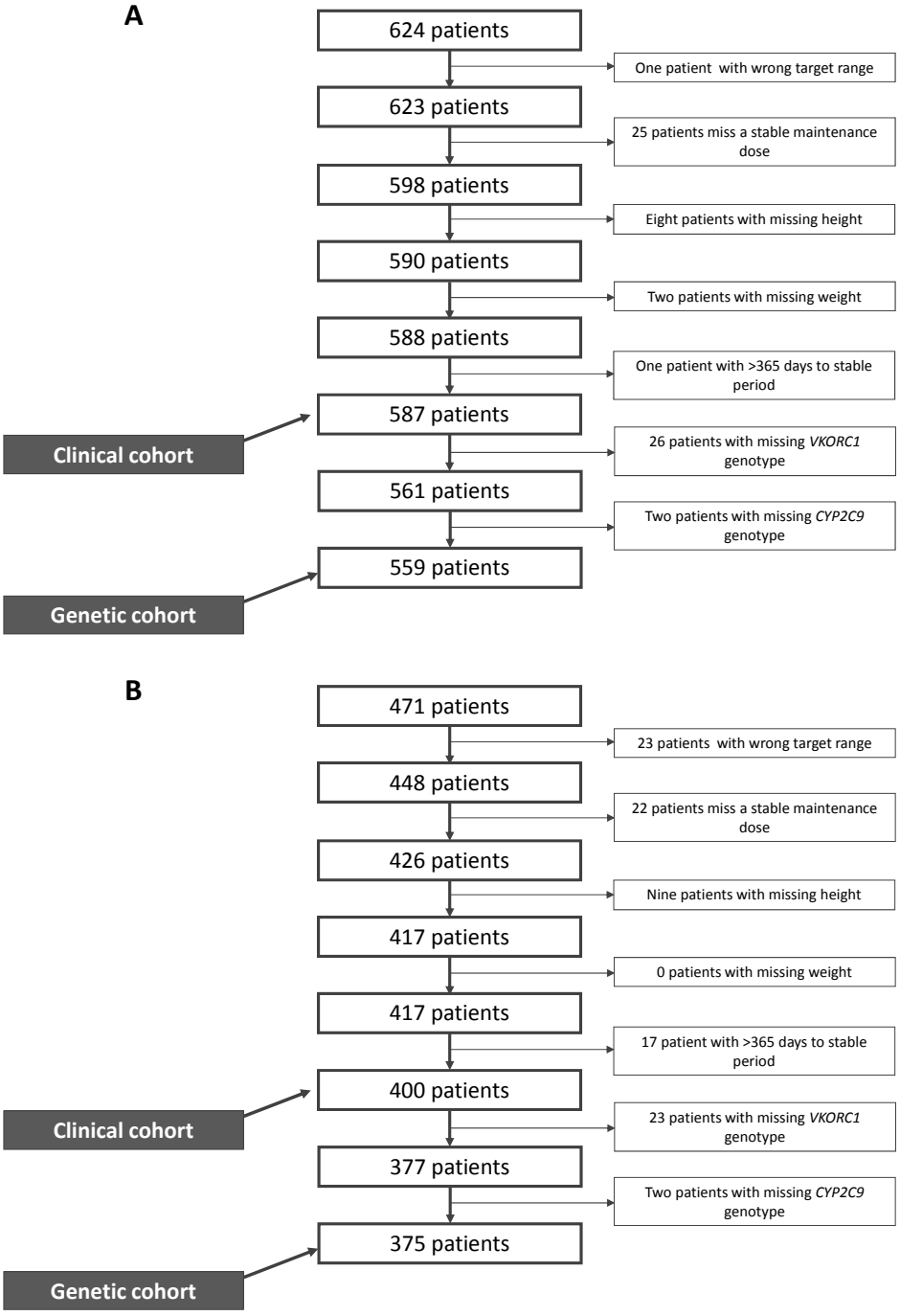

Figure 1. Flowcharts of patients included in the phenprocoumon cohort (A) and the acenocoumarol cohort (B).

Table 1. Characteristics of patients treated with acenocoumarol and phenprocoumon[a].

	Phenprocoumon cohort	Acenocoumarol cohort
Patient characteristics		
Age in years[b]	71.4 (44.6-88.2)	74.7 (51.4-87.5)
Male sex[c]	338 (57.6)	224 (56.0)
Height in cm[b]	172 (153-192)	172 (154-195)
Weight in kg[b]	80 (52-120)	79 (52-125)
Use of Amiodarone[c]	27 (4.6)	9 (2.2)
Maintenance dose in mg per day[b]	2.12 (0.83-4.27)	2.34 (1.00-5.00)
INR during first stable period[b]	2.71 (2.08-3.44)	2.61 (2.00-3.49)
Genetic factors		
CYP2C9 genotype[c]		
*1/*1	406 (67.9)	292 (65.9)
*1/*2	105 (17.6)	84 (19.0)
*1/*3	60 (10.0)	48 (10.8)
*2/*2	11 (1.8)	8 (1.8)
*2/*3	10 (1.7)	7 (1.6)
*3/*3	3 (0.5)	2 (0.5)
Unable to determine genotype	3 (0.5)	2 (0.5)
No blood available[d]	26	28
VKORC1 genotype[c]		
CC	230 (38.5)	155 (35.0)
CT	279 (46.7)	225 (50.8)
TT	87 (14.5)	63 (14.2)
Unable to determine genotype	2 (0.3)	0 (0)
No blood available[d]	26	28

[a] For the patient characteristics, data of patients used for the development of the nongenotype-guided algorithm are reported (n=587 for phenprocoumon and n=400 for acenocoumarol). For the genetic factors, data of all included patients are reported (n=624 for phenprocoumon and n=471 for acenocoumarol).
[b] Presented is median (2.5^{th}-97.5^{th} percentile)
[c] Presented are numbers of patients (%)
[d] In 4.2% (phenprocoumon) and 5.9% (acenocoumarol) of the cases, no blood was available

to a given maintenance dose for acenocoumarol. Briefly, the predicted loading dose is rounded off to the highest number of tablets equivalent to the estimated dose, whereas for the subsequent days the dose is divided equally.

External validation of phenprocoumon & acenocoumarol algorithms

External validation of the genotype-guided algorithms without height and weight yielded an R^2 of 59.4% and a mean absolute error of 0.46 mg/day for phenprocoumon and an R^2 of 49.0% and a mean absolute error of 0.57 mg/day for acenocoumarol. For

Table 2. Algorithms for phenprocoumon and acenocoumarol[a].

	Phenprocoumon			Acenocoumarol		
	Genotype-guided	Nongenotype-guided	Univariate R^2 (%) on the sqrt(dose)	Genotype-guided	Nongenotype-guided	Univariate R^2 (%) on the sqrt(dose)
Intercept	2.874	1.652		4.117	2.635	
CYP2C9 genotype			4.6			4.5
*1/*1	0[b]	-		0[b]	-	
*1/*2	-0.259	-		-0.093	-	
*1/*3	-0.342	-		-0.519	-	
*2/*2	-0.447	-		-0.435	-	
*2/*3	-0.684	-		-0.466	-	
*3/*3	-0.681	-		-1.375	-	
VKORC1 genotype			34.1			27.2
CC	0[b]	-		0[b]	-	
CT	-0.601	-		-0.572	-	
TT	-1.394	-		-1.267	-	
Age, in years	-0.015	-0.011	8.1	-0.027	-0.027	14.1
Sex, if female	0.026	0.105	2.1	0.271	0.386	0.2
Height, in cm	0.011	0.011	7.3	0.009	0.013	6.3
Weight, in kg	0.008	0.013	12.8	0.010	0.013	11.8
Amiodarone use, if yes	-0.345	-0.343	0.5	-0.377	-0.167	0.2
Unadjusted R^2 of the algorithm	55.9%	17.3%		52.6%	23.7%	

[a] The outcome is the square root of the mean first stable maintenance dose in mg/week for the INR target range 2.0-3.5. If the target range 2.0-3.0 is used, all coefficients need to be divided by sqrt(1.07).
[b] The value of this parameter is zero because it is the reference group.
Note: The formula for, for example, the genotype-guided algorithm of phenprocoumon should be read as: Square root mean maintenance dose (mg/week)= 2.874 – 0 (if CYP2C9*1/*1) – 0.259 (if CYP2C9*1/*2) – 0.342 (if CYP2C9*1/*3) – 0.447 (if CYP2C9*2/*2) – 0.684 (if CYP2C9*2/*3) – 0.681 (if CYP2C9*3/*3) – 0 (if VKORC1 CC) – 0.601 (if VKORC1 CT) – 1.394 (if VKORC1 TT) – 0.0153 * age (years) + 0.026 (if female) + 0.0113 * height (cm) + 0.0085 * weight (kg) – 0.345 (if amiodarone is used).

the nongenotype-guided algorithm, the R^2 was 23.5% (phenprocoumon) and 17.8% (acenocoumarol) and the mean absolute error was 0.62 mg/day (phenprocoumon) and 0.72 mg/day (acenocoumarol). Figure 3 shows plots of predicted vs. observed maintenance dose in the validation sets. For results of the validation using multiple imputation, see Supplementary material online.

Table 3. Loading doses for phenprocoumon as derived from the individual maintenance dose[a].

Dose day 1 (mg)	Dose day 2 (mg)	Dose day 3 (mg)	Maintenance dose range (mg per day)
3	3	3	<1.04
6	3	3	1.04-1.31
6	6	3	1.31-1.61
6	6	6	1.61-1.85
9	6	6	1.85-2.92
9	9	6	>2.92[b]

[a] The lower limit of the maintenance dose range corresponds with the given loading dose, e.g. a loading regimen of 6-3-3 leads to a monitoring dose of 1.04 mg/day.
[b] Only for genotype-guided algorithm.

Table 4. Loading doses for acenocoumarol as derived from the individual maintenance dose[a].

Dose day 1 (mg)	Dose day 2 (mg)	Dose day 3 (mg)	Maintenance dose range (mg per day)
1	1	1	<1.00
2	1	1	1.00-1.25
2	2	1	1.25-1.75
2	2	2	1.75-2.00
3	2	2	2.00-2.25
3	3	2	2.25-2.75
3	3	3	2.75-3.00
4	3	3	3.00-3.25
4	4	3	3.25-3.75
4	4	4	3.75-4.00
5	4	4	4.00-4.25
5	5	4	4.25-4.75
5	5	5	4.75-5.00

[a] The lower limit of the maintenance dose range corresponds with the given loading dose.

Discussion

Several genotype-guided algorithms have previously been developed for warfarin[12-16]. However, algorithms to estimate the phenprocoumon and acenocoumarol maintenance dose are only developed in small cohorts (<100 patients) and are non-validated[17,18]. In this paper, we present genotype-guided and nongenotype-guided algorithms for the determination of the loading and maintenance phase of phenprocoumon and acenocoumarol treatment based on data derived from almost 1000 patients. The nongenotype-guided algorithms estimate the individual patient dose requirement for initiation of coumarin therapy based on patient characteristics

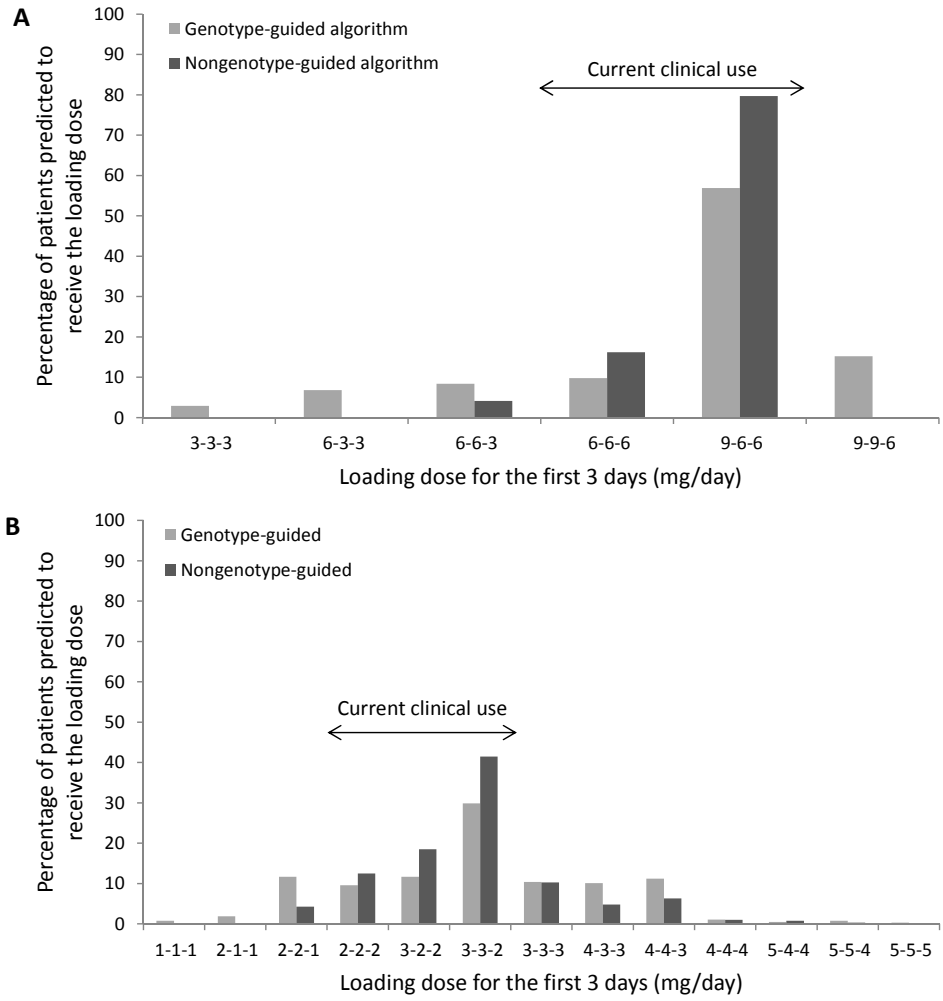

Figure 2. Predicted distribution of loading dose in the phenprocoumon cohort (A) and the acenocoumarol cohort (B). The arrow indicates the currently used clinical loading dose.

of age, sex, height, weight, and amiodarone use. The genotype-guided algorithms include *CYP2C9* and *VKORC1* genotype additional to the variables included in the nongenotype-guided algorithms. These algorithms are thought to be an improvement compared with the current clinical situation, where each patient receives a standard loading dose which is subsequently adjusted to the individual dose with the INR. However, this hypothesis needs to be tested in a randomized controlled trial.

Even though novel anticoagulants have recently entered the market, phenprocoumon and acenocoumarol are anticipated to remain commonly prescribed anticoagu-

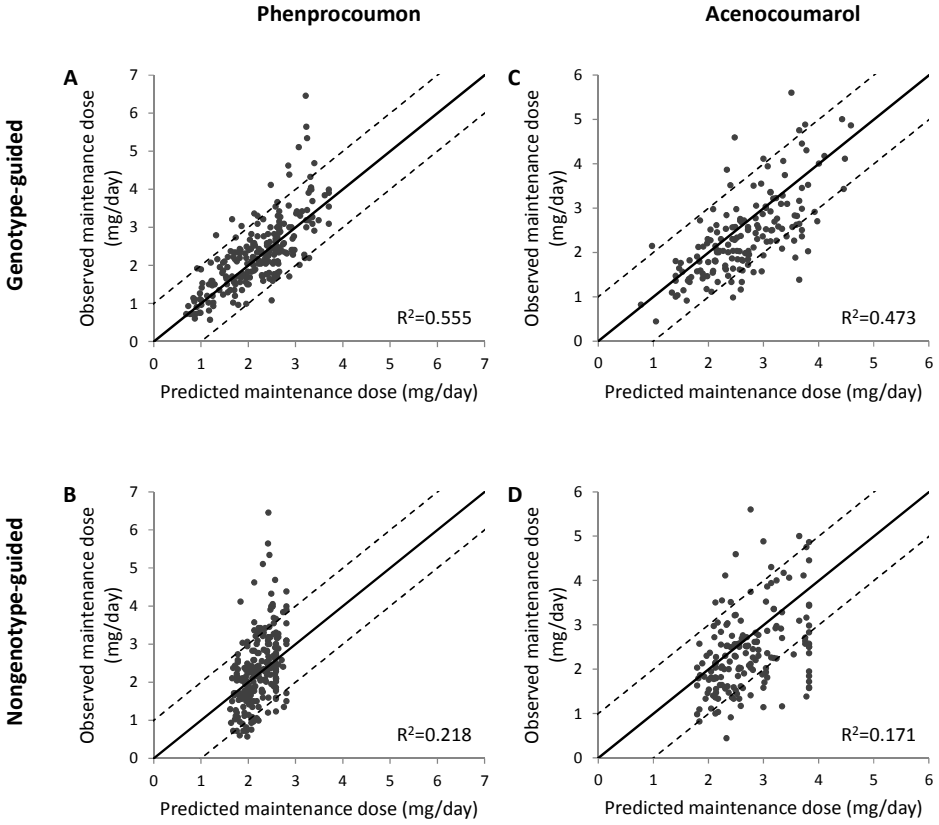

Figure 3. Predicted maintenance dose vs. observed maintenance dose in the validation data set for phenprocoumon (left column) and acenocoumarol (right column) for both the genotype-guided (upper row) and nongenotype-guided algorithms (lower row), p =0.05 for all four graphs. The black line represents the perfect prediction model. The dashed lines are the perfect prediction lines ±1.0 mg/day.

lants because new anticoagulants are more expensive and have no antidote, and the experience is limited at the moment. To avoid increased costs, it is likely that authorities will encourage continued use of coumarin derivatives. Therefore, it is crucial to improve the safety of the treatment with coumarin derivatives. Randomized clinical trials are needed to investigate whether the safety of the anticoagulation treatment will improve by developing individualized dosing regimens as shown in this paper.

Before the anticoagulant therapy starts, the maintenance dose can be calculated by filling in the algorithm. Then the loading dose belonging to the calculated maintenance dose can be found in Table 3 (phenprocoumon) and Table 4 (acenocoumarol). For example, a 78-year-old man who starts phenprocoumon therapy, with weight of

91 kg, height 180 cm, and genotype *CYP2C9*1/*2* and *VKORC1* CT, who does not use amiodarone would need a maintenance dose of 1.80 mg/day. The maintenance dose was calculated as follows: square root of the mean weekly dose in mg = 2.874–0.259 (*CYP2C9*1/*2*) - 0.601(*VKORC1 CT*) - 0.015* 78 (age) + 0.011 * 180 (height) + 0.008*91 (weight) = 3.55√mg/week. The week dose in milligram is 3.55^2 = 12.62 mg/week, which equals 1.80 mg/day. This maintenance dose corresponds (Table 3) to a loading dose of 6 mg on day 1, 6 mg on day 2, and 6 mg on day 3.

Both square root and log transformations are common for the warfarin algorithms[12-17]. We used the square root transformation of the maintenance dose since this was on average the best outcome measurement (see Supplementary material online). Explained variability of our algorithms are comparable with earlier developed warfarin algorithms and to the small cohort acenocoumarol algorithm[12-16]. In addition, the correlations between various prediction scores for the maintenance dose given in the literature and our two algorithms are high (see Supplementary material online), showing similarity between our algorithms and the earlier developed warfarin algorithms.

Based on theory, it could be expected that polymorphisms in the *CYP2C9* gene influence the maintenance doses less for phenprocoumon than for acenocoumarol. However, it was shown in this paper that the effects of these polymorphisms are comparable for phenprocoumon and acenocoumarol; *CYP2C9* explains 4.6 and 4.5% of the dose variability, respectively. It is supported by other studies that polymorphisms in the *CYP2C9* gene does influence phenprocoumon doses; some studies found lower phenprocoumon doses or increased bleeding risks[26-28] for patients having an SNP in the *CYP2C9* gene, where that of Visser *et al*.[29] did not.

We have considered the use of the *CYP2C9* genotype to individualize the elimination half-life of coumarins to calculate the genotype-specific accumulation indexes. However, the available data of the *CYP2C9* effect on the elimination half-lives are too limited[30] to be used in our algorithm, and additional assumptions would have to be made. In addition, the *CYP2C9* genotype is already used as a parameter to calculate the maintenance dose, which is used to derive the loading dose. Therefore, *CYP2C9* genotype is indirectly used to estimate the loading dose.

Our study has some limitations. First, some bias might have been introduced. We collected data from current phenprocoumon and acenocoumarol users. This approach may introduce some selection bias because long term users are more likely to be selected. However, the distribution of allele frequencies, amiodarone use, sex,

and age is similar as in cohorts of other studies[13,16,17]. Furthermore, we had no data about patient compliance and non-compliance and that could be a source of bias. Nevertheless, non-compliance would reduce the R^2 of the algorithms, because it dilutes the effects. Therefore, we anticipate that the selection and compliance bias have a minor effect. Secondly, it is possible that the estimated height and weight are slightly off due to errors in these variables. Thirdly, we did not include drugs other than amiodarone use, ethnicity, smoking status, and diet as factors in the algorithm, although other drugs have been shown to affect dose requirements in some studies. Interacting drugs other than amiodarone were not significantly associated with the maintenance dose or did not increase the explained dose variation and therefore were not included in the algorithms. The main reason that ethnicity, smoking status, and diet were excluded from the algorithms is because these factors are challenging to assess accurately and objectively. Our aim was to develop a clinically applicable algorithm with the most important determinants that can be easily implemented in a routine care setting.

The strengths of this study are that the algorithms were developed using large patient populations and that the algorithms performed equally well when validated in independent prospective data sets.

The aim of this study was to develop genotype-guided and nongenotype-guided algorithms to determine the maintenance dose of phenprocoumon and acenocoumarol, and to derive the individualized loading algorithms for phenprocoumon. The question of whether the use of these algorithms will improve clinical care will be answered in the upcoming EU-PACT trial, a two-armed, single-blinded, randomized controlled trial taking place in six European countries[31]. The main outcome of the trial will be whether a pharmacogenetics-guided algorithm increases the time into the therapeutic range during the first 3 months of therapy. In addition, the cost-effectiveness of pretreatment genotyping will be assessed looking at adverse events, i.e. thromboembolic events and hemorrhages, and the quality-adjusted life-years.

Supplementary material

Supplementary material is available at European Heart Journal online: http://eurheartj.oxfordjournals.org/content/32/15/1909/suppl/DC1.

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Chapter 4

Validation of the acenocoumarol EU-PACT algorithms: similar performance in the Rotterdam Study cohort as in the original study

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Abstract

Aim

To evaluate the performance of the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) acenocoumarol dose algorithms in an independent data set. The EU-PACT trial investigates the added value of pretreatment genotyping for use of warfarin, phenprocoumon and acenocoumarol.

Patients & methods

External validation was performed in the Rotterdam Study cohort using information about 707 acenocoumarol users. R^2, which measures the strength of correlation between the predicted and observed acenocoumarol dose, mean absolute error and mean squared error were calculated to evaluate the performance of the original algorithm.

Results

Validation resulted in a R^2 of 52.7% and 12.9% compared with an R^2 of 52.6% and 17.8% in the original study for the genotype-guided and nongenotype-guided dose algorithm, respectively. For the genotype-guided dose algorithm, the mean absolute error was 0.48 mg/day and the mean squared error was 0.38 $(mg/day)^2$. For the nongenotype-guided dose algorithm, the mean absolute error was 0.62 mg/day and the mean squared error was 0.63 $(mg/day)^2$.

Conclusion

The EU-PACT acenocoumarol algorithm performs just as accurately in this study as in the original study, which implies applicability in various populations.

Introduction

Coumarins are effective medications for treating and preventing thrombosis. However, these drugs have a small therapeutic window and there is wide intra- and inter-individual variability in dose requirements[1, 2]. Therefore, the use of coumarin derivatives is associated with drug-related hospitalization[3-6]; patients receiving coumarin therapy are at risk of thrombosis and hemorrhage[7, 8]. Therefore, the therapeutic response to coumarins should be monitored in order to keep the International Normalized Ratio (INR) within boundaries. Several patient characteristics, such as weight and age, contribute to the variation in dose requirements. However, the largest contribution to the dose variation appears to be due to genetic factors. Approximately 35–50% of the dose variation is explained by SNPs in the genes that encode for the metabolizing enzyme CYP2C9, which plays a major role in the metabolism of the coumarins, and by a SNP in the *VKORC1* gene, which is the therapeutic target of the coumarins[9].

In recent years, the development of algorithms to predict a personalized coumarin dose has gained interest. These algorithms support physicians to make individual coumarin dose decisions and thereby potentially reduce the risk of over- and underdosing. Many dose algorithms have been developed, especially for warfarin, which is the main coumarin prescribed in the USA[10-14]. Less attention has been paid to acenocoumarol and phenprocoumon, which are the coumarins of first choice in continental Europe[15-17]. Three studies provided dose algorithms, of which two studies only provided dose algorithms based on small patient cohorts and were not externally validated[16, 17]. The third study describes how the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) dose algorithms[15] were developed, which are based on large patient populations and were externally validated in independent data sets[18, 19]. A genotype-guided and a nongenotype-guided dose algorithm were developed and validated[15]. However information about height and weight were missing in the independent validation data sets.

The aim of this study was to evaluate the performance of the EU-PACT genotype-guided and nongenotype-guided acenocoumarol dose algorithms in an independent data set in which data on height and weight are available. The added value of genotyping before the start of coumarin therapy is investigated in the EU-PACT trial[20].

Patients & methods

Study design & patients

Data used for the analysis were obtained from the original basis cohort of the Rotterdam Study[21-23]. This prospective population-based cohort study among 7983 persons was designed to investigate disease occurrence in a population aged over 45 years, including cardiovascular diseases. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. The approval has been renewed every 5 years.

For this study, we selected all patients with a stable acenocoumarol maintenance dose who had a therapeutic range between 2.0 and 3.5. This is the maintenance dose in the first stable period defined as a period of at least 3 weeks with three or more consecutive INR measurements within therapeutic range (INR: 2.0–3.5) with less than 10% change in the acenocoumarol dose. Regarding the clinical cohort in which the nongenotype-guided dose algorithm was validated, patients were excluded if they had at least one missing value for height, weight or age. Regarding the genetic cohort, which was used to validate the genotype-guided dose algorithm, the same exclusion criteria were used as for the clinical cohort, and in addition patients with a missing *VKORC1* or *CYP2C9* genotype were excluded.

Data collection & genotyping

All cohort members of the Rotterdam Study had baseline examinations with completed standardized questionnaires, sampling blood and isolation of DNA and several examinations. Additional information, such as baseline weight and baseline height, were collected at the study center. All cohort members of the Rotterdam Study, treated with coumarins, are monitored by a regional anticoagulation clinic, the Star Medical Diagnostic Centre. From this clinic, since 1984, all dosing, laboratory, and clinical data are fully computerized[24]. 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.

PCR followed by restriction enzyme digestion analysis were used to genotype for *CYP2C9*2* and *CYP2C9*3* allele variants. All detected allelic variants were reanalyzed[25, 26]. For genotyping *VKORC1*, the *1173C>T* SNP was chosen, which is in very high linkage disequilibrium with *-1639G>A* in all races[27, 28]. Genomic DNA was extracted from peripheral venous blood samples[29]. A total of 1–2 ng DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH300 pipetting robot (Caliper LS). Taq-

man® allelic discrimination assays were used to perform the genotyping[29]. *CYP2C9*1* and *VKORC1 C* genotypes were assigned if variant alleles in the analyzed corresponding SNPs (*CYP2C9*2*, *CYP2C9*3* and *VKORC1 1173C>T*) were lacking. Other variant alleles are rare in Caucasians. Therefore, there is a negligible risk for misclassification of phenotypes due to other variant alleles. Of the randomly selected samples, 5% were re-genotyped with the same method as quality control. No inconsistencies were observed.

Outcome & determinants

The square root of the mean stable coumarin maintenance dose in mg/day during the first stable period was used as the outcome measure for both the genotype-guided dose algorithm and the nongenotype-guided dose algorithm. The square root of the dose was taken in order to obtain a normal distributed outcome measure. The *a priori* defined determinants included in the EU-PACT dose algorithms were *CYP2C9* and *VKORC1* genotypes, age in years, sex, amiodarone use, height in cm and weight in kg. The genotype-guided and nongenotype-guided dose algorithms are shown in Box 1.

Box 1. Genotype-guided and nongenotype-guided dose algorithm

- Genotype-guided square root mean maintenance dose (mg/week) = 4.117 - 0 (if CYP2C9*1/*1) - 0.093 (if CYP2C9*1/*2) - 0.519 (if CYP2C9*1/*3) - 0.435 (if CYP2C9*2/*2) - 0.466 (if CYP2C9*2/*3) - 1.375 (if CYP2C9*3/*3) - 0 (if VKORC1 CC) - 0.572 (if VKORC1 CT) - 1.267 (if VKORC1 TT) - 0.027 × age (years) + 0.271 (if female) + 0.009 × height (cm) + 0.010 × weight (kg) - 0.377 (if amiodarone is used)

- Nongenotype-guided square root mean maintenance dose (mg/week) = 2.635 - 0.027 × age (years) + 0.386 (if female) + 0.013 × height (cm) + 0.013 × weight (kg) - 0.167 (if amiodarone is used)

Statistical analysis

It was assessed whether genotypes were in Hardy–Weinberg equilibrium using the χ^2 test. The EU-PACT dose algorithms were used to calculate for each patient in the Rotterdam Study a predicted maintenance dose. The predicted maintenance dose was compared with the actual observed maintenance dose by calculating the R^2 between the predicted maintenance dose and the observed maintenance dose, the mean squared error and the mean absolute error. This was carried out for both the genotype-guided and the nongenotype-guided dose algorithms. SPSS statistics version 19.0 was used for all analysis.

Results

Patient cohort

In the Rotterdam Study cohort, 1616 patients used a coumarin, of which 1450 used acenocoumarol. Of these patients, 854 patients had a therapeutic range of 2.0–3.5. After selection of patients with complete data for the *a priori* determinants and did not need more than 365 days to reach a stable maintenance dose, 707 acenocoumarol users remained for the validation of the nongenotype-guided dose algorithm and for the validation of the genotype-guided dose algorithm, 628 acenocoumarol patients remained (Figure 1). Approximately 99% of the patients were Caucasian. Patient characteristics are summarized in Table 1.

Table 1. Characteristics of patients treated with acenocoumarol in the Rotterdam Study cohort.

	Rotterdam study cohort (n=707)
Patient characteristics	
Age in years[a]	77 (62-90)
Male sex[b]	253 (35.8%)
Height in cm[a]	166 (150-186)
Weight in kg[a]	75 (54-99)
Use of amiodarone[b]	16 (2.3)
Maintenance dose in mg per day[a]	2.19 (0.85-4.31)
INR during first stable period[a]	2.7 (2.0-3.5)
Genetic factors	
CYP2C9 genotype[b,c]	
*1/*1	434 (61.4)
*1/*2	133 (18.8)
*1/*3	59 (8.3)
*2/*2	8 (1.1)
*2/*3	10 (1.4)
*3/*3	-
Missing[e]	63
VKORC1 genotype[b,d]	
CC	237 (33.5)
CT	318 (45.0)
TT	79 (11.2)
Missing[e]	73

[a] Presented is median (2.5^{th}-97.5^{th} percentile)
[b] Presented are numbers of patients (%)
[c] Hardy-Weinberg Equilibrium *CYP2C9*: χ^2=2.574, p=0.46.
[d] Hardy-Weinberg Equilibrium *VKORC1*:χ^2=3.070, p=0.08.
[e] Missing genotypes were due to missing blood samples (approximately 80%) and genotype failure (approximately 20%).

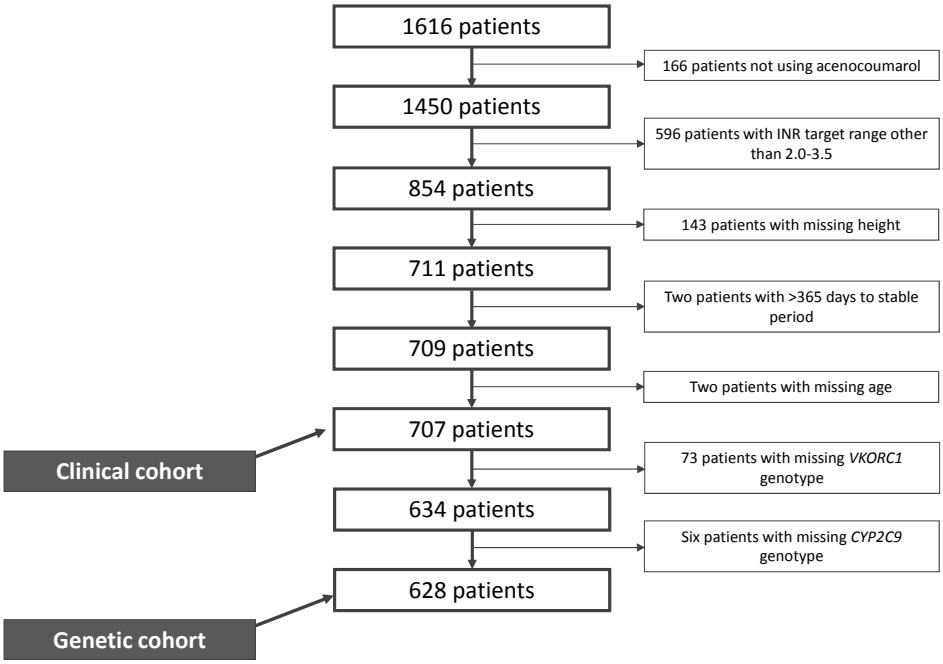

Figure 1. Patients included in the analysis. INR stands for International Normalized Ratio.

All genotype distributions followed Hardy–Weinberg equilibrium. Allele frequencies for *CYP2C9* were 0.82 for the wild-type allele, 0.12 for *CYP2C9*2* and 0.05 for *CYP2C9*3*. Allele frequencies for *VKORC1 1173C>T* were 0.62 for C and 0.38 for T, which is in accordance with other Caucasian populations.

External validation of acenocoumarol algorithms

External validation of the genotype-guided acenocoumarol algorithm yielded an R^2 of 52.7% for the square root dose, a mean absolute error of 0.48 mg/day and a mean squared error of 0.38 $(mg/day)^2$. For the nongenotype-guided acenocoumarol algorithm, the R^2 was 12.9% for the square root dose, the mean absolute error was 0.62 mg/day and the mean squared error was 0.63 $(mg/day)^2$. Figures 2 & 3 show predicted versus observed maintenance dosages in the Rotterdam Study cohort.

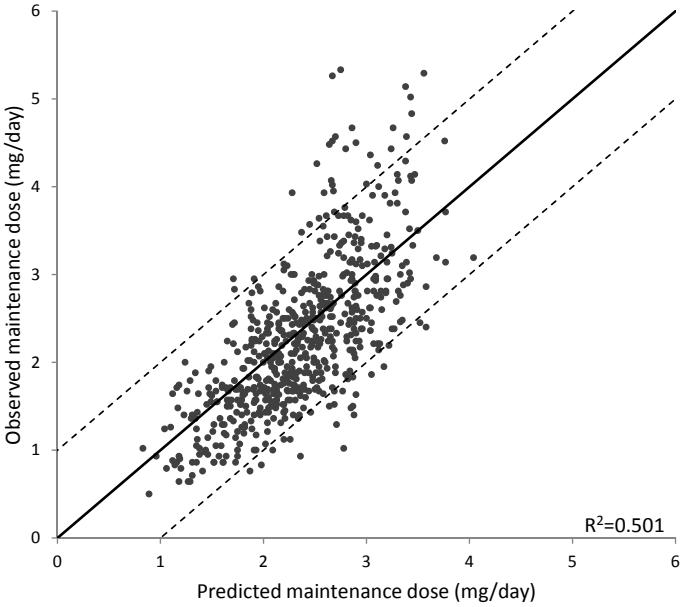

Figure 2. Predicted maintenance dose versus observed maintenance dose in the Rotterdam Study cohort for the genotype-guided acenocoumarol dose algorithm. With a population of 707 patients, a power of 90% and an α of 0.05, it is possible to detect correlations of 0.113 or higher, corresponding to an R^2 of 0.013 or higher. Since all R^2 values were higher than 0.013, it could be concluded that $p < 0.05$. The solid line represents the perfect prediction model. The dashed lines are the perfect prediction line ±1.0 mg/day.

Discussion

This study shows that the EU-PACT acenocoumarol algorithms[15] perform equally in the independent Rotterdam Study cohort[21-23] as in the original study. This means that the algorithms are valid in another Dutch population, and application of the algorithm is not restricted to the anticoagulation center where the algorithm was developed. This suggests that the EU-PACT algorithms are robust and valid for different Dutch populations.

The EU-PACT acenocoumarol algorithms[15] were validated in this study using information on height and weight. These two parameters were not available for the external validation of the algorithms in the data set of Schalekamp *et al*.[18]. Our study showed an R^2 of 52.7 and 12.9% for the genotype-guided and nongenotype-guided dose algorithms, respectively. External validation in the data set of Schalekamp *et al*. resulted in an R^2 of 49.0 and 17.8% for the genotype-guided and nongenotype-dose algorithms, respectively[15]. It is remarkable that inclusion of height and weight led

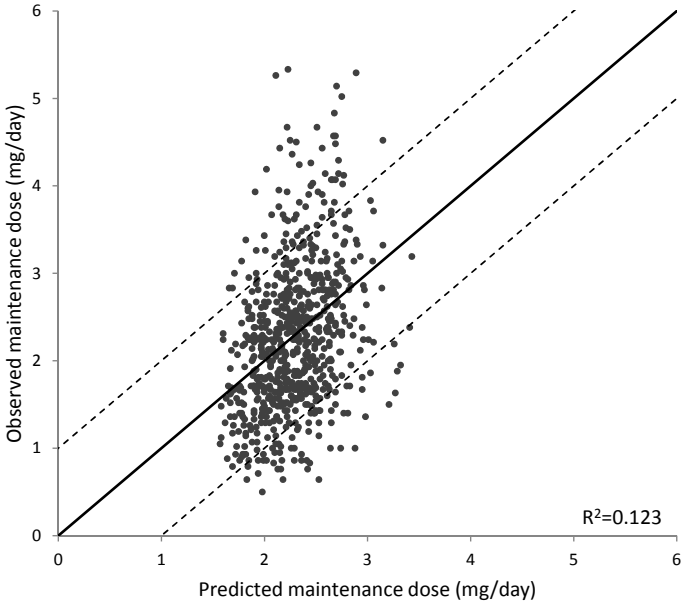

Figure 3. Predicted maintenance dose versus observed maintenance dose in the Rotterdam Study cohort for the nongenotype-guided acenocoumarol dose algorithm. With a population of 707 patients, a power of 90% and an α of 0.05, it is possible to detect correlations of 0.113 or higher, corresponding to an R^2 of 0.013 or higher. Since all R^2 values were higher than 0.013, it could be concluded that $p < 0.05$). The solid line represents the perfect prediction model. The dashed lines are the perfect prediction line ±1.0 mg/day.

to a lower R^2 in the nongenotype-guided dose algorithm. This could be caused by population differences. The Rotterdam Study cohort contains older -2 years for the Pre-EU-PACT data set and 10 years for the Schalekamp *et al*. data set- and less male patients than the cohorts used in the original study[15, 18]. However, for the nongenotype-guided dose algorithm the mean absolute error and the mean squared error in the Rotterdam Study were smaller than the validation in the Schalekamp data set and in the original Pre-EU-PACT data set in which the algorithm was developed. Values for the mean squared error were 0.63, 0.84 and 0.86 $(mg/day)^2$ for the Rotterdam Study cohort, Pre-EU-PACT cohort and Schalekamp cohort, respectively. Values for the mean absolute error were 0.62, 0.69 and 0.72 mg/day for the Rotterdam Study cohort, Pre-EU-PACT cohort and Schalekamp cohort, respectively.

From the graphs, it looks as if the algorithm underestimates observed doses at higher doses. The reason is that safety guidelines were built in to the model – that is, truncated weight, height and age. Furthermore, still approximately 40% of the dose variability is not yet explained.

The weakness of this study is that the Rotterdam Study cohort was also collected in The Netherlands. The results of the genotype-guided dose algorithm are good, which implies that this dose algorithm perform well in the Dutch population, but it is still uncertain how it performs in populations in other countries. In The Netherlands, patients are intensively guided by anticoagulation clinics, which might provide better anticoagulant care[30]. Also, factors such as a different diet, weight and height might influence dose requirements in other countries. Most patients in the studies are Caucasians (99%). We therefore expect that the algorithms will be valid in other Caucasian populations as well.

We provided additional validation results that show that the EU-PACT acenocoumarol algorithms are applicable in multiple Dutch populations, making them suitable to predict the maintenance dose of patients starting acenocoumarol anticoagulant therapy. Based on the validation, we are not able to predict clinical outcomes. The algorithms validated in this manuscript are currently being tested in the EU-PACT trial[20]. Improvements in safety and efficacy due to pretreatment genotyping as well as cost–effectiveness will be investigated. Results are expected to become available in 2013.

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Chapter 5

Genotype-guided dosing of coumarin derivatives: the European pharmacogenetics of anticoagulant therapy (EU-PACT) trial design

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Abstract

The narrow therapeutic range and wide interpatient variability in dose requirement make anticoagulation response to coumarin derivatives unpredictable. As a result, patients require frequent monitoring to avert adverse effects and maintain therapeutic efficacy. Polymorphisms in *VKORC1* and *CYP2C9* jointly account for about 40% of the inter-individual variability in dose requirements. To date, several pharmacogenetic-guided dose algorithms for coumarin derivatives, predominately for warfarin, have been developed. However, the potential benefit of these dosing algorithms in terms of their safety and clinical utility has not been adequately investigated in randomized settings. The European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial will assess, in a single-blinded and randomized controlled trial with a follow-up period of 3 months, the safety and clinical utility of genotype-guided dosing in daily practice for the three main coumarin derivatives used in Europe. The primary outcome measure is the percentage time in the therapeutic range for international normalized ratio. This report describes the design and protocol for the trial.

Introduction

Coumarin derivatives, such as warfarin, acenocoumarol and phenprocoumon, are commonly prescribed as oral anticoagulants for the treatment of thromboembolic disorders. Therapy with coumarin derivatives is most effective when the international normalized ratio (INR) is kept within a narrow range. Prescribing of these drugs is difficult because of their narrow therapeutic window and the wide inter-individual variability in dose requirement; coumarin derivative dosages can vary by a factor 10 among patients[1, 2]. For these reasons, it is difficult to predict anticoagulation response to a standard dosing regimen, as this is the case with the current dosing algorithms used for the initiation of anticoagulant therapy. Consequently, treatment is often either subtherapeutic (due to underdosing) or supratherapeutic (due to overdosing), placing the patient at risk of (recurrent) thrombosis or hemorrhage, respectively, which can be life-threatening[3, 4]. Because of the uncertainty in anticoagulation response, patients on coumarin derivative therapy require careful monitoring[1, 4, 5].

Anticoagulation response to coumarin derivatives is influenced by a number of clinical, environmental and genetic factors. It has been established that factors such as concurrent drug therapy, co-morbidity, age, sex, BMI, smoking and dietary vitamin K intake influence coumarin derivative dose requirements[6-10]. Polymorphisms in the *CYP2C9* and *VKORC1* genes, encoding for the metabolizing enzyme cytochrome P450 2C9 (CYP2C9) and the target enzyme vitamin K epoxide reductase (VKOR) respectively, together account for about 40% of the variability in coumarin derivative maintenance dose requirements[11, 12]. Several studies have demonstrated that patients with allelic variants in the *CYP2C9* and *VKORC1* genes require lower coumarin derivatives doses than those with wild-type alleles[13-17]. This exposes them to a greater risk of overanticoagulation and hemorrhage, particularly during initiation of therapy. Polymorphisms in other genes, for example, the *CYP4F2* gene, have only a nominal effect on the coumarin derivate dose[18, 19].

The recognition that genetic factors influence coumarin derivative dose requirements and thus may predispose to serious and life threatening hemorrhage has highlighted the inadequacy of the currently used dosing regimens. To date, several studies have quantified the contribution of the *CYP2C9* and *VKORC1* genes in coumarin derivative dose requirement and put forward pharmacogenetic-based dosing equations[10, 12, 20-25]. However, these equations (mainly developed for warfarin) are based on data derived from patients on stable maintenance therapy and are therefore unsuitable for those commencing oral anticoagulant therapy, where loading doses often are used. The challenge in demonstrating the benefits of pharmacogenetic guided dosing lies in the

development of dosing algorithms, which can improve the accuracy of dosing during both the initiation of therapy and subsequent maintenance therapy. A pharmacogenetic approach to oral anticoagulant therapy requires two things: robust dosing algorithms that allow for prediction of loading and maintenance doses developed from verification data and validated in a replication set; and also that the clinical validity and utility of the dosing algorithms is tested within a randomized controlled trial (RCT) setting. The cost–effectiveness of genotype-guided dosing must also be examined, since current available cost–effectiveness analyses (CEA) do not point in the same direction[26-28]. Additional clinical trials are necessary to prove the clinical relevance and cost–effectiveness of pretreatment genotyping before implementing this approach in clinical practice.

The European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial will assess the safety, clinical utility and cost–effectiveness of the newly developed pharmacogenetic-guided dosing algorithms for acenocoumarol, phenprocoumon and warfarin. The trial will take place in seven countries in Europe and is supported by the European Commission FP7 Programme.

Patients & methods

Design & setting

The EU-PACT study is a two-armed, single blinded (patients are blinded), RCT which will test the effectiveness of dosing regimens that include genetic factors compared with dosing regimens without these factors. This will be evaluated during the first 3 months after initiation of anticoagulant therapy in patients treated with the three different coumarin derivatives warfarin, acenocoumarol, or phenprocoumon. The study will be performed in 13 different centers, covering general practitioners, anticoagulation clinics and hospitals. These centers are located in seven European countries, namely the UK, Sweden, the Netherlands, Spain, Greece, Germany and Austria. It will take 2 years to complete the inclusion of patients.

Sample size calculation

Based on data from the first 3 months of warfarin therapy in Sweden and the UK, the mean percentage time in range (%TIR) of the INR ranges from 52 to 65% in large cohorts of patients with atrial fibrillation (AF) or venous thrombosis (VT). In other participating centers, similar values for acenocoumarol and phenprocoumon are found. For example, in the Netherlands, where predominately acenocoumarol and phenprocoumon are used, the average range of %TIR of the INR across differ-

ent anticoagulation clinics varies from 22.5 to 56.5% (median 38.0%) for the first 8 weeks of coumarin derivative therapy and from 32.5 to 67.0% (median 53.5%) in patients anticoagulated short-term (2–6 months therapy)[29]. With 80% power and at 5% significance level, a total of 442 patients each in the intervention and control groups will be needed in order to demonstrate a 5% greater %TIR of the INR. Assuming a 10% dropout after study entry, 985 patients will need to be recruited for each coumarin derivative, making a total of 2955 patients randomized for the three trials.

Study population

Newly diagnosed patients with either AF or VT, that is, pulmonary embolism (PE) or deep vein thrombosis (DVT), requiring anticoagulant therapy with acenocoumarol, phenprocoumon or warfarin are eligible for the trial if they meet the inclusion and exclusion criteria.

Inclusion & exclusion criteria

Patients of both sexes, aged 18 years or older, diagnosed with AF or VT requiring coumarin derivative therapy with a target INR in the lower intensity range (2.0–3.5 in the Netherlands and 2.0–3.0 in the other participating countries) for at least 12 weeks are eligible for the trial. They must have the ability to attend the scheduled visits and have to provide written informed consent.

Patients will be excluded from the trial if they have been treated with a coumarin derivative previously, if their *CYP2C9* or *VKORC1* genotype is known, if they are pregnant or breastfeeding, or if they suffer from severe cognitive impairment. The presence of a mechanical heart valve will also lead to exclusion. Another exclusion criterion is an abnormal clotting function at baseline INR, that is a baseline INR of 1.5 and higher, a platelet count less than 100×10^9 L^{-1} or an activated partial thromboplastin time (APTT) more than 1.3 times upper reference value that is not explained by the presence of lupus anticoagulants.

Patient allocation & treatment

Patients will be randomized to either the intervention group, which will be dosed according to a genotype-guided dosing algorithm, or to the control group, which will be dosed according to a dosing regimen without genotype. All dosing regimens will be computer assisted. The genotype-guided dosing algorithm will include the patient's genetic information, clinical and demographic data and in the monitoring phase the previous INR. The acenocoumarol and phenprocoumon control groups will be dosed according to a nongenotype-guided dosing algorithm, which uses the

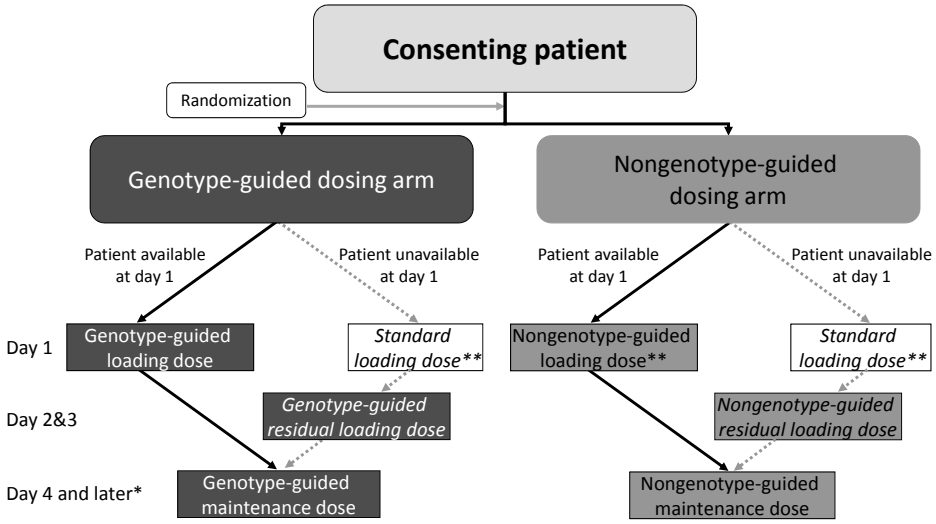

Figure 1. Schematic presentation of the study design as for each coumarin derivative.
*The loading dose algorithm is optimally followed for 3 days, and the start of the monitoring dose algorithm on day 4. However, due to weekends the monitoring dose algorithm may also start on day 3, 5 or 6.
**In exceptional cases (shown by the dashed arrow), if the patient is randomized to either the intervention or the control group later than day 1, and the patient has received coumarin dosing according to usual clinical care only at day 1, the patient may receive a loading dose according to one of the loading dose algorithms for the two days remaining until the next planned international normalized ratio test.

same parameters of the dosing algorithm in the intervention group except for the *CYP2C9* and *VKORC1* genotypes. The warfarin control group will be dosed according to standardized clinical care. A schematic presentation of the study design is given in Figure 1.

The dosing algorithms for all three coumarin derivatives will be developed with various data sets[30, 31] which contain information about factors known to influence dose requirements such as age, sex, *CYP2C9* and *VKORC1* genotype, height and weight. All dosing algorithms are divided into two subalgorithms; the loading dose algorithm and the monitoring dose algorithm. The monitoring dose algorithms for acenocoumarol and phenprocoumon will be developed with linear regression. The warfarin monitoring dose algorithm is based on the model developed by the International Warfarin Pharmacogenetics Consortium[21]. Loading doses for all three coumarin derivatives will be calculated from the monitoring dose using pharmacokinetic information of the coumarin derivative for each genotype.

Blinding & randomization

The EU-PACT trial is a single-blinded study with patients being blinded to the study treatment. Staff with access rights to the database will be able to view all data on patients recruited in their own center, except for genotype in the control patients.

Eligible patients who consent to take part in the study will undergo medical screening prior to enrollment. Patient demographics and clinical data including age, sex, height, weight and information on co-morbidity (e.g., malignancies and thyroid disease), comedication and alcohol intake will be recorded. Following a successful screen and fulfillment of the study inclusion criteria, patients will be randomized to the intervention or control group by block randomization per study center.

Patient consent & information

All patients will be informed verbally and in writing about the aims of the study and how participation would affect their treatment. Standard information about the impact of diet (such as vitamin K intake and alcohol) and interacting drugs (e.g., CYP2C9 inducers and inhibitors) and the possible hazards associated with the therapy will be provided to all patients.

Genotyping

A blood sample will be taken from all participating patients for genotyping of *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910) and *VKORC1 -1639G>A* (rs9923231) prior to the commencement of oral anticoagulant therapy. Genotyping will be performed using a new rapid method which provides results within 1.5 hours. The method employs a HyBeacon® technology (LGC Ltd, Middlesex, UK)[32-34], which will be used in combination with Optigene's Genie 1 instrument (Optigene Ltd, Horsham, UK) as a point-of-care test in a non-laboratory environment. An aliquot of blood will be stored for quality control analysis of the point-of-care test.

The blood samples from patients who are found to be either sensitive or resistant to a coumarin derivative will later be subjected to further genetic analysis through sequencing approaches for the identification of rare mutations in genes mediating the pharmacology or disposition of coumarin derivatives and other novel genes to identify the genetic basis of discordant phenotypes[35].

Treatment procedures

Patients suffering from VT who require acute anticoagulation will initially be treated with (parenteral) low molecular weight heparin in combination with the coumarin derivative. Low molecular weight heparin will be discontinued according to local

guidance. Patients with AF initiating anticoagulation for stroke prophylaxis will be administered coumarin derivatives alone.

The loading dose will be calculated according to the loading dose algorithm for the prescribed coumarin derivative. If a patient for some reason does not receive the individualized starting dose (e.g., because of weekends), (s)he can obtain an adjusted loading dose regimen on day 2. After the first INR determination (preferably on day 4), subsequent dosing will be calculated according to the monitoring dose algorithm. The monitoring dose algorithm is based on the same factors as the loading dose algorithm including the patient's previous INR.

The use of all concurrent medications will be recorded, including the drug's trade name, dosage, start and end date, and indication for treatment.

Alcohol intake, quality of life & compliance assessment

Alcohol intake (alcohol use disorders identification test; AUDIT)[36] and quality of life (EQ-5D)[37] will be assessed at the patient's first and last study visits. To monitor patient compliance to treatment, patients will complete a medication questionnaire at each visit.

Assessment of safety

International normalized ratio measurements will be carried out on days 1, 4, 6, 8, 15, 22, 57 and 85 (days 4–85 may be adjusted slightly) to ensure that patients are adequately anticoagulated. If clinically needed, additional INR measurements will be performed.

Any undesired medical event, not necessarily related to the use of coumarin derivatives, is defined as a serious adverse event if it leads to death; is life-threatening, for example, a major hemorrhage; requires (prolonged) hospitalization or is a congenital anomaly/birth defect[38]. Hemorrhages will be categorized into major and minor according to the International Society on Thrombosis and Haemostasis (ISTH) classification of hemorrhagic events[39]. The algorithm of Naranjo *et al*. will be used to determine the probability that any observed (serious) adverse event is associated with the trial treatment[40]. Safety data will be evaluated by an independent Data and Safety Monitoring Board (DSMB).

Patient withdrawal

At any time, patients are free to withdraw from the trial without giving a reason. The patient will be contacted to obtain information about the reason(s) for withdrawal

and any experienced adverse events (AEs). The date and reason for the withdrawal will be reported in the case report form (CRF). Each patient withdrawn less than 2 weeks after study entry will be replaced by a new one.

The investigator will be able to withdraw patients from the trial for safety reasons, for example, due to AEs that contraindicate continued participation, pregnancy or a deteriorated general condition.

After participation in the trial, anticoagulant therapy for each patient will be continued according to individual needs and local protocols.

Ethics

The EU-PACT study will be performed according to the study protocol, International Conference on Harmonisation Good Clinical Practice (ICH-GCP), the Declaration of Helsinki, EU directives and applicable regulatory requirements. The study will be submitted for approval to medical review ethics committees in all participating countries.

Study outcomes

Primary outcomes

The primary outcome of the study is the %TIR of the INR, INR range 2.0–3.0, during the first three months following initiation of anticoagulant therapy calculated by the interpolation method[41].

Secondary outcomes

The secondary outcomes for the first 3 months of therapy include:

- Time to and number of patients with INR 4.0 or above, which indicates overanticoagulation;
- Percentage of time spent with INR 4.0 or above;
- Percentage of time spent with INR 1.5 or less, which indicates underanticoagulation;
- Time to reach therapeutic INR defined as the time to the first INR within target range, provided that a subsequent INR measured at least 1 week later is also within target range;
- Time to reach stable dose defined as time to reach an unchanged dose (<10% change) at consecutive visits and the INR being within the target range for a period of at least 3 weeks;

- Time to and number of minor and major hemorrhages;
- Time to and number of thromboembolic events;
- The occurrence of coumarin derivative hypersensitivity defined as dose requirements of 1.5 mg warfarin/day or less, 1.5 mg phenprocoumon/day or less or 1.0 mg acenocoumarol/day or less during maintenance;
- The occurrence of coumarin derivative resistance defined as dose requirements of at least 10 mg warfarin/day, at least 6 mg phenprocoumon/day or at least 8 mg acenocoumarol/day during maintenance;
- Number of coumarin derivative dose adjustments;
- The utility of the LGC Ltd's rapid genotyping test in daily anticoagulation practice;
- Patient quality of life;
- The cost-effectiveness of pharmacogenetic-guided dosing for each of the three coumarin derivatives.

Data collection

Information on each patient's age, sex, height, weight, alcohol intake, co-morbidity (e.g., malignancies and thyroid disease), and comedication will be obtained either directly from the initial interview with the patient or the self-completed questionnaires and from medical records or pharmacy records whenever possible. Other parameters including INR values, number of INR measurements, coumarin derivative doses, the number of dose changes, changes in concurrent disease or drug therapy, laboratory data and any adverse events experienced during the study will be recorded during the course of the trial.

Data management

Data collection and storage will be done using Promasys (Promasys, Leiden, The Netherlands), a data management software system which supports multicenter trials. The central functionality of this system is related to data management, which allows the setting up of a database structure into which the clinical trial data can be entered thereby preserving the integrity of the data captured. Each center can log on to the central server and enter data live in the electronic CRF.

Statistical analysis

The trial results will be evaluated according to the intention-to-treat and the per-protocol analysis. Estimation of mean differences (plus 95% CIs) in %TIR of the INR between genotype and nongenotype-guided dosing, when necessary with correction for confounding variables, will be performed with linear regression for each coumarin derivative. For the remaining outcomes, appropriate hypothesis tests will be adopted to test for differences between the two study arms, for example the Cox-proportional hazard model for dichotomous outcomes. These outcomes will also be presented using Kaplan-Meier curves. The nominal p-value for assessing statistical significance will be 0.05, although this will be adjusted for the number of comparisons made in order to conserve the type I error rate. All hypothesis tests will be two-sided. We will analyze whether the results found are similar for all three anticoagulation drugs and whether they are similar within and between the different study centers. For the latter, multilevel regression models stratified by center will be used. To be able to extrapolate our data to the whole EU these comparisons are of the utmost importance.

Cost-effectiveness analysis

A CEA for the individualized dosing regimen will be performed according to established methods[42]. The primary analysis will utilize the societal perspective, meaning that all costs will be included in the CEA regardless of who incurs these costs. As a consequence, we will include not only health service costs (e.g., from ambulatory care, hospital care and medications), but also costs incurred by patients. Two types of health outcomes will be examined: the incidence of adverse events (hemorrhage, thromboembolic events); and the quality-adjusted life-years (QALYs), measured using the EQ-5D. Two sets of CEA will be performed: a short-term CEA and a long-term CEA. The short-term CEA will focus on the clinical results during the study's 3 month follow-up period. We will perform a long-term CEA, which will be possible by creating a model that combines the results of this RCT with data from other clinical, epidemiological, and health services research studies. We plan to analyze coumarin derivative-specific and country-specific data to estimate resource use and unit costs.

Discussion

Current strategies for initiation of oral anticoagulant therapy are inadequate, exposing patients to a risk of (recurrent) thrombosis owing to underdosing or hemorrhage owing to overdosing. Polymorphisms in *CYP2C9* and *VKORC1* genes have a major impact on coumarin derivative dose requirements. It is anticipated that pharmacogenetic-guided dosing will improve the safety of anticoagulant therapy with coumarin derivatives through improved accuracy of dosing. The three small scale prospective studies reported to date have not convincingly demonstrated the potential benefit of pharmacogenetic-guided dosing on treatment outcomes[43-45]. One of these studies included both *CYP2C9* and *VKORC1* polymorphisms[44] whilst the other two only included polymorphisms in *CYP2C9* in the dosing algorithms used[43, 45]. Inclusion of the *VKORC1* gene in a pharmacogenetic-guided dosing algorithm is likely to improve the accuracy of dosing given that *VKORC1* polymorphisms explain up to a third of the inter-individual variability in coumarin derivative dose requirements. Moreover, reported ongoing randomized trials of genotype-guided dosing as well as the aforementioned small prospective studies only evaluate pharmacogenetic-guided dosing with warfarin[46]. In contrast, the EU-PACT trial will be evaluating a pharmacogenetic approach to anticoagulant therapy with the three most prescribed coumarin derivatives in large patient cohorts in seven European countries using pharmacogenetic-based dosing algorithms which include both *CYP2C9* and *VKORC1* genes. The EU-PACT trial results should therefore be widely applicable to patients across Europe and elsewhere. Unlike previous prospective studies, the EU-PACT trial will make use of rapid point-of-care genotyping, thus making it possible to commence genotype-guided therapy straight away.

It is anticipated that initiation of anticoagulant therapy using a genotype-guided dosing regimen allows the patient to reach target INR quickly and to remain within the therapeutic range more effectively. Although hemorrhages are clinically more relevant than percentage time within target INR, we chose %TIR rather than hemorrhages as the primary study outcome. It would not be financially and logistically possible to collect the considerably larger number of patients needed in order to detect a difference in hemorrhages. It is well established that maintaining anticoagulation within the therapeutic range is critical for therapeutic efficacy and safety. Several studies have shown that there is a close relationship between the INR and the risk of hemorrhage and thrombotic events; the risk of hemorrhage increases markedly for supratherapeutic INR values[47-49], and the risk of death owing to cerebral hemorrhage doubles for every unit increase in INR[50], while the risk of a thrombotic event increases with subtherapeutic INR values[4, 48]. Because of this close association between INR and

these outcomes, it is expected that our chosen primary outcome is a good indicator of treatment safety outcome.

The goal of this study is to evaluate the added value and the cost–effectiveness of pharmacogenetic-guided dosing of coumarin derivatives in daily practice. To our knowledge, EU-PACT is the first large scale randomized controlled trial of pharmacogenetic-guided anticoagulant therapy ever performed in Europe.

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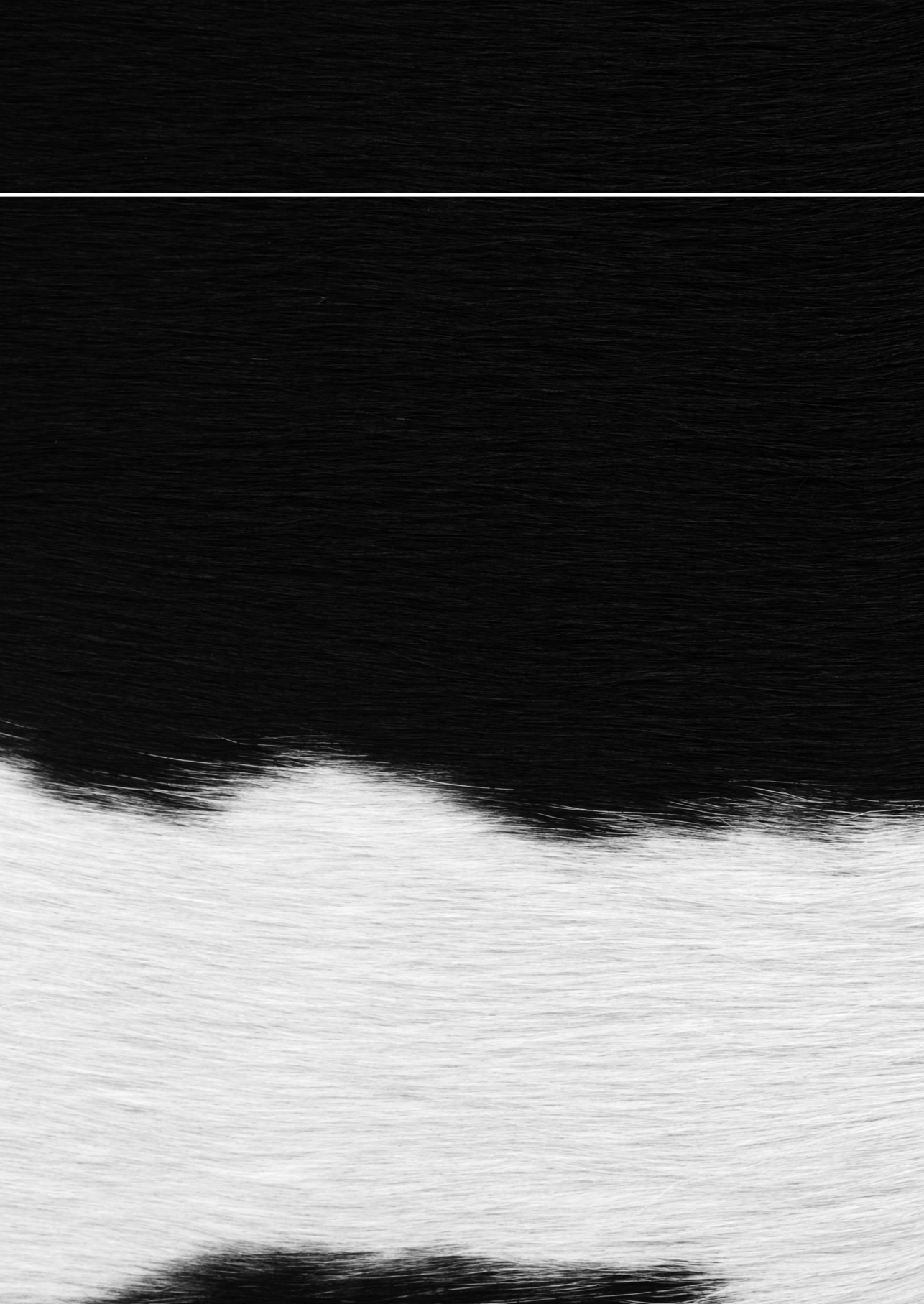

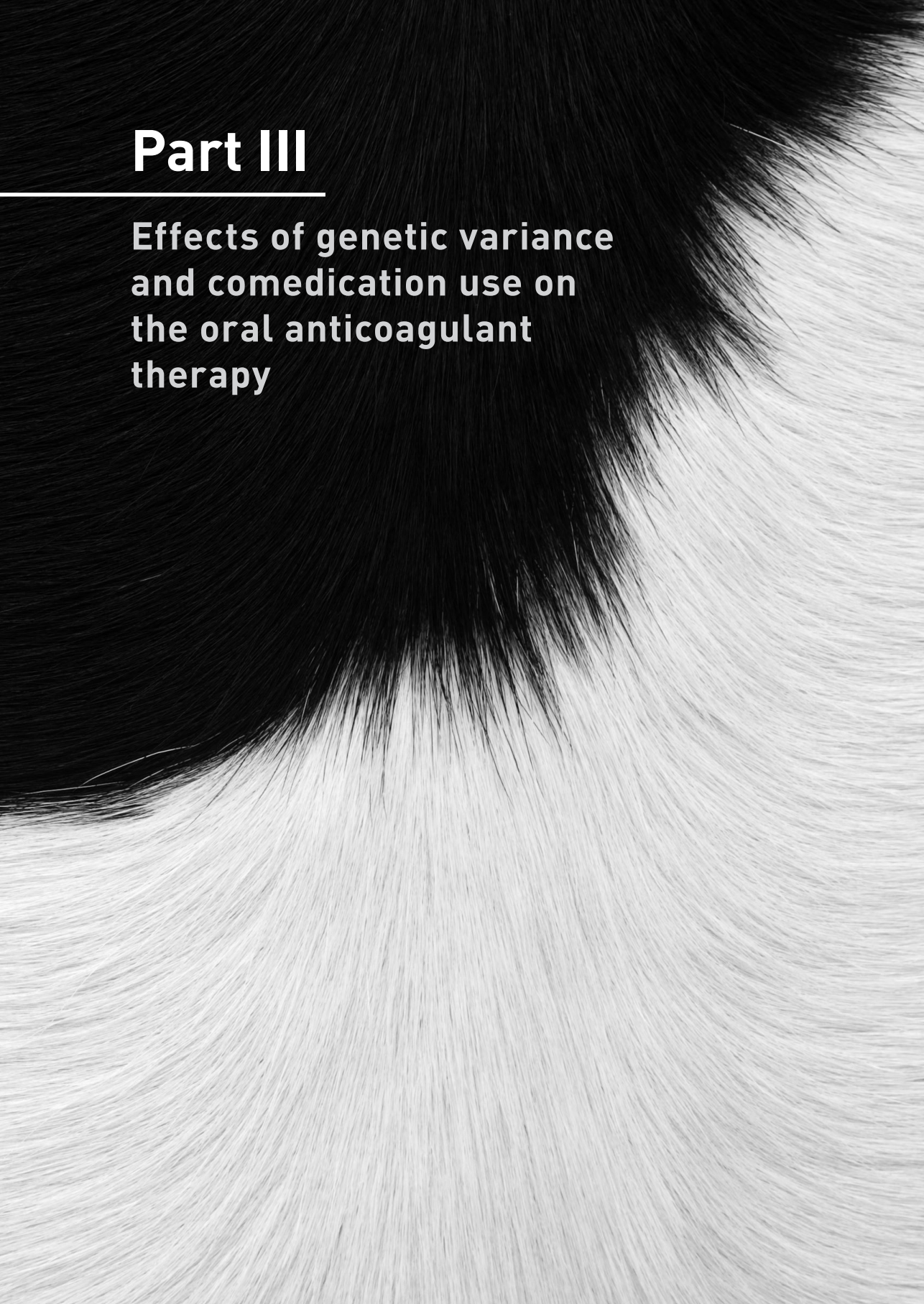

Part III

Effects of genetic variance and comedication use on the oral anticoagulant therapy

Chapter 6

An evaluation of gene-gene interaction between the *CYP2C9* and *VKORC1* genotypes affecting the anticoagulant effect of phenprocoumon and acenocoumarol

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Abstract

Aim

Previous studies have provided contradictory results regarding the interaction between the *CYP2C9* and *VKORC1* genotypes affecting various outcome measures. We aimed to provide a definite answer regarding the question whether there exists a gene-gene interaction between the *CYP2C9* and *VKORC1* genotypes affecting the anticoagulant effect of phenprocoumon and acenocoumarol.

Patients & methods

The EU-PACT cohort data set, which contains data on 624 phenprocoumon and 471 acenocoumarol patients, was used. Patient characteristics, pharmacogenetic data, International Normalized Ratios (INRs) and dosages were available. We investigated whether there was an interaction between the *CYP2C9* and *VKORC1* genotypes affecting the maintenance dose, time to severe overanticoagulation, and time to achieve stability during the first 180 days of phenprocoumon and acenocoumarol therapy, in addition to the effect of the separate genotypes. The interaction effect was investigated by adding the product term of the *CYP2C9* and *VKORC1* genotype classes for four different commonly used *CYP2C9* classifications to the linear regression model – for the outcome measure maintenance dose – or to the Cox regression models – for the outcome measures time to severe overanticoagulation and time to achieve stability.

Results

No significant interactions -all p-values above 0.23 for phenprocoumon and 0.30 for acenocoumarol- were observed for all outcome measures.

Conclusion

There are no interactions between the *CYP2C9* and *VKORC1* genotypes affecting the maintenance dose, time to severe overanticoagulation, and time to achieve stability for phenprocoumon and acenocoumarol.

Introduction

Coumarins have a narrow therapeutic window, and there is wide inter- and intra-individual variability in dose requirements[1, 2]. Coumarin users are at risk of thrombosis, owing to underdosing, or at risk of hemorrhage, owing to overdosing[3, 4]. This frequently results in drug-related hospitalization[5-7]. Therefore, patients are monitored by measurement of the International Normalized Ratio (INR) followed by dose adjustment if necessary.

Patient characteristics such as age and body size influence the dose requirements of coumarins. Genetic factors, notably polymorphisms in the vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*) and the cytochrome P450 2C9 gene (*CYP2C9*) together explain 35–50% of the inter-individual variability in dose requirements[8, 9]. Factors such as *VKORC1* and *CYP2C9* genotype, age, weight, height and medication use are commonly used in dosing algorithms for coumarins[9-11]. Only a few studies have investigated a possible interaction between the *CYP2C9* and *VKORC1* genotypes in addition to the effects of the separate genotypes[9, 12-15]. Contradictory results on different outcome measures, such as maintenance dose, severe overanticoagulation, and time to achieve stability, have been found (Table 1). In addition, genetic variation in *CYP2C9* is categorized differently among different studies. Therefore, this study aimed to investigate a possible interaction effect between the *VKORC1* and *CYP2C9* genotypes, in addition to the effects of the separate genotypes, for different categorizations of *CYP2C9* and on three different outcome measures during the use of phenprocoumon or acenocoumarol: maintenance dose, severe overanticoagulation, and time to achieve stability. In contrast to the previous studies that investigated the interaction effect between the *VKORC1* and *CYP2C9* genotypes, this study investigated the interaction effect for three different outcome measures and four different *CYP2C9* classifications. This gives rise to 12 different combinations, and therefore provides a complete overview of results for both phenprocoumon and acenocoumarol.

Patients & methods

Data from the pre-EU-PACT cohort study were used for these analyses, and a more detailed description has been published elsewhere[9]. Patients currently using either phenprocoumon or acenocoumarol were eligible to take part in the study if they were aged 18 years and over and had a target INR in the lowest intensity category (according to Dutch guidelines: INR 2.0– 3.5). Pregnant or lactating women, patients

Table 1. Overview of interaction studies between the *CYP2C9* and *VKORC1* genotypes.

Coumarin	Outcome measure	Significant interaction observed?	Reference
Warfarin	Dose	No	11
Acenocoumarol	Time to severe overanticoagulation	Yes	12
	Time to achieve stability	No	
Phenprocoumon	Maintenance dose	Yes	13
	Time to severe overanticoagulation	No	
	Time to achieve stability	No	
Acenocoumarol	Acenocoumarol response	No	14
Acenocoumarol	INR & risk of severe overanticoagulation	No	15
	Mean dosage at end of the initiation period	Yes	

who were in a nursing home and patients participating in other clinical studies were excluded. Eligible patients who had a scheduled visit at the anticoagulation clinic from either 10 to 12 November 2009 (Anticoagulation Clinic Leiden, phenprocoumon) or from 23 to 27 November 2009 (Anticoagulation Clinic Medial, acenocoumarol) were invited to participate. Height, current weight (weight at the moment of inclusion) and weight at the start of the anticoagulant therapy were recorded for each participant. Data on the participants' age, sex, history of comedication, history of INR values and prescribed coumarin doses were obtained from the electronic registry databases of the anticoagulation clinics. Procedures were in accordance with the Helsinki Declaration, and all patients gave their informed consent. The Medical Ethics Committee Leiden approved the study protocol.

Genotyping

Residual blood samples from INR measurements were used to genotype the patients for *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), and *VKORC1 1173C>T* (rs9934438), with predesigned Taqman assays (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), and according to the manufacturer's protocol. *CYP2C9*1* and *VKORC1 C* genotypes were assigned if polymorphisms in the analyzed corresponding Single Nucleotide Polymorphisms (*CYP2C9*2*, *CYP2C9*3*, and *VKORC1 1173C>T*) were lacking. Other variant alleles are rare in Caucasians. Therefore, there is a negligible risk of misclassification of phenotypes because of other variant alleles. Genotypes were determined on a Light-Cycler 480 (Roche Diagnostics, Almere, The Netherlands) in 384-well plates that include positive (previously established genotype) and negative (Tris-EDTA buffer) controls. In addition, as a quality control, 10% of the samples were genotyped in duplicate.

Outcome measure

We investigated three outcome measures:

1. Maintenance dose in milligrams per day in the first stable period after initiation of anticoagulant therapy. A stable period was defined as a period of at least 3 weeks with ≥3 consecutive INR measurements within the target range with < 10% change in the coumarin dose.
2. Time to severe overanticoagulation, defined as the time to first INR of > 6.0.
3. Time to achieve stability, defined as the time to the first dose of the stable period after initiation of the anticoagulant therapy.

Statistical analysis

To investigate the interaction between the *CYP2C9* and *VKORC1* genotypes, the product term of the gene classes was added to the linear regression model – the outcome measure was the square root of the maintenance dose- or to the Cox regression model -for the time to severe overanticoagulation and time to reach stability- in addition to the independent genotype classifications. Also, *a priori* defined determinants were used as covariates for this model, which were equal to the determinants used in our dosing algorithms; age in years, sex, amiodarone use, height in centimeters, and weight in kilograms[9]. Patients with missing values for at least one of the above-mentioned determinants or those who did not reach a stable period within a year following the onset of the therapy -only applicable for the outcome measure maintenance dose- were excluded from the analysis. For patients without an INR > 6.0 or who did not achieve stability, the event-time was censored at the maximum follow-up period. If weight at the start of the anticoagulation treatment was missing, the current weight was used instead. We used one categorization with three classes for the *VKORC1* genotype, namely homozygote wild-type, and heterozygote and homozygote variant allele. The *CYP2C9* genotype was categorized in four different ways:

1. Six classes: *1/*1, *1/*2, *2/*2, *1/*3, *2/*3, and *3/*3
2. Two classes: only *1/*1 and the rest (i.e. *1/*2, *1/*3, *2/*2, *2/*3, and *3/*3)
3. Three classes: only *1/*1 and *2 (i.e. *1/*2 and *2/*2) and the rest (i.e. *1/*3, *2/*3, and *3/*3)
4. Three classes: only *1/*1 and at least one *2 (i.e. *1/*2, *2/*2, and *2/*3) and the rest (i.e. *1/*3 and *3/*3).

To investigate the effect of the interaction between the *CYP2C9* and *VKORC1* genotypes on the maintenance dose, we added the genotype classes and the product terms of the *CYP2C9* genotype classes and the *VKORC1* genotype to the genotype-guided phenprocoumon and acenocoumarol linear regression models[9]. The smallest

data set -acenocoumarol users- contained enough patients to show a dose difference of 0.035 mg daily with 80% power and a significance level of 0.05. In the same way, main terms and the product terms were added to the Cox regression analysis. The acenocoumarol data set contained enough patients to detect an increase or decrease in hazard ratio of at least 0.29 for time to achieve stability and 0.23 for time to severe overanticoagulation with 80% power and a significance level of 0.05. In the phenprocoumon set, it would be possible to detect an increase or decrease in the hazard ratio of 0.15 for time to achieve stability and of 0.19 for time to severe overanticoagulation. All analyses were performed with adjustment for age, sex, height, weight, and amiodarone use[9]. Statistical significance was defined as $p<0.05$. For the analysis, we used the statistical software SPSS (PASW Statistics, Armonk, NY, USA) version 18.

Results

Patient cohort

The total data set contained information about 624 phenprocoumon users and 471 acenocoumarol users[9]. We excluded patients with missing *CYP2C9* or *VKORC1* genotype information, weight, or height, or a wrong target range. Therefore, we used data of 583 phenprocoumon users and 413 acenocoumarol users. As the therapy start date was not available for 48 phenprocoumon users and 122 acenocoumarol users, data of 535 phenprocoumon users and 291 acenocoumarol users were available for study of the interaction between the *CYP2C9* by *VKORC1* genotypes for the outcome measures time to severe overanticoagulation and time to achieve stability in the first 180 therapy days. For the maintenance dose, data of 559 phenprocoumon users and 375 acenocoumarol users were available, as not all patients achieved a stable maintenance dose. The flowchart is given in the Supplementary material online. More men than women were included in the study, and the mean ages of the patients were 71.5 and 74.7 years for phenprocoumon and acenocoumarol, respectively (Table 2). The *CYP2C9* and *VKORC1* genotypes were in Hardy-Weinberg equilibrium[9].

Interaction

No interaction between the *CYP2C9* and *VKORC1* genotypes was found in this study (Figure 1). Figure 1 displays the results for one of the four *CYP2C9* classifications, namely three classes for the *CYP2C9* genotype: only *1/*1 and *2 (i.e. *1/*2 and *2/*2) and rest (i.e. *1/*3, *2/*3, and *3/*3). The results for the remaining three classifications were similar. For the dose, all graphs, as shown in Figure 1, were paral-

Table 2. Characteristics of patients treated with phenprocoumon and acenocoumarol[a].

	Phenprocoumon cohort	Acenocoumarol cohort
Patient characteristics		
Age in years[b]	71.5 (43.9-88.5)	74.7 (51.9-87.4)
Male sex[c]	340 (58.3)	229 (55.4)
Height in cm[b]	172 (153-192)	172 (154-193)
Weight in kg[b]	80 (52-120)	80 (51-120)
Use of Amiodarone[c]	26 (4.5)	10 (2.4)
Genetic factors		
CYP2C9 genotype[c]		
*1/*1	406 (67.9)	292 (65.9)
*1/*2	105 (17.6)	84 (19.0)
*1/*3	60 (10.0)	48 (10.8)
*2/*2	11 (1.8)	8 (1.8)
*2/*3	10 (1.7)	7 (1.6)
*3/*3	3 (0.5)	2 (0.5)
Unable to determine genotype	3 (0.5)	2 (0.5)
No blood available	26	28
VKORC1 genotype[c]		
CC	230 (38.5)	155 (35.0)
CT	279 (56.7)	225 (50.8)
TT	87 (14.5)	63 (14.2)
Unable to determine genotype	2 (0.3)	0 (0)
No blood available	26	28

[a] For the patient characteristics age, sex, height, weight and use of amiodaron, data of 583 phenprocoumon users and 413 acenocoumarol users are presented. For the genetic factors, data of all included patients are reported (n=624 for phenprocoumon and n=471 for acenocoumarol). In 4.2% (phenprocoumon) and 5.9% (acenocoumarol) of the cases, no blood was available.
[b] Presented is median (2.5^{th}-97.5^{th} percentile)
[c] Presented are numbers of patients (%)

lel for each genotype classification, meaning that there was no interaction (Figure 1A,B). For the time to severe overanticoagulation and time to achieve stability, hazard ratios are presented for the *CYP2C9* and *VKORC1* genotype combinations (Figure 1C–F), showing no interaction between the *CYP2C9* and *VKORC1* genotypes. P-values for the interaction terms were not significant for all outcomes, all genotype classifications, and both coumarins -all p-values were above 0.23 for phenprocoumon and 0.30 for acenocoumarol (see Supplementary material online).

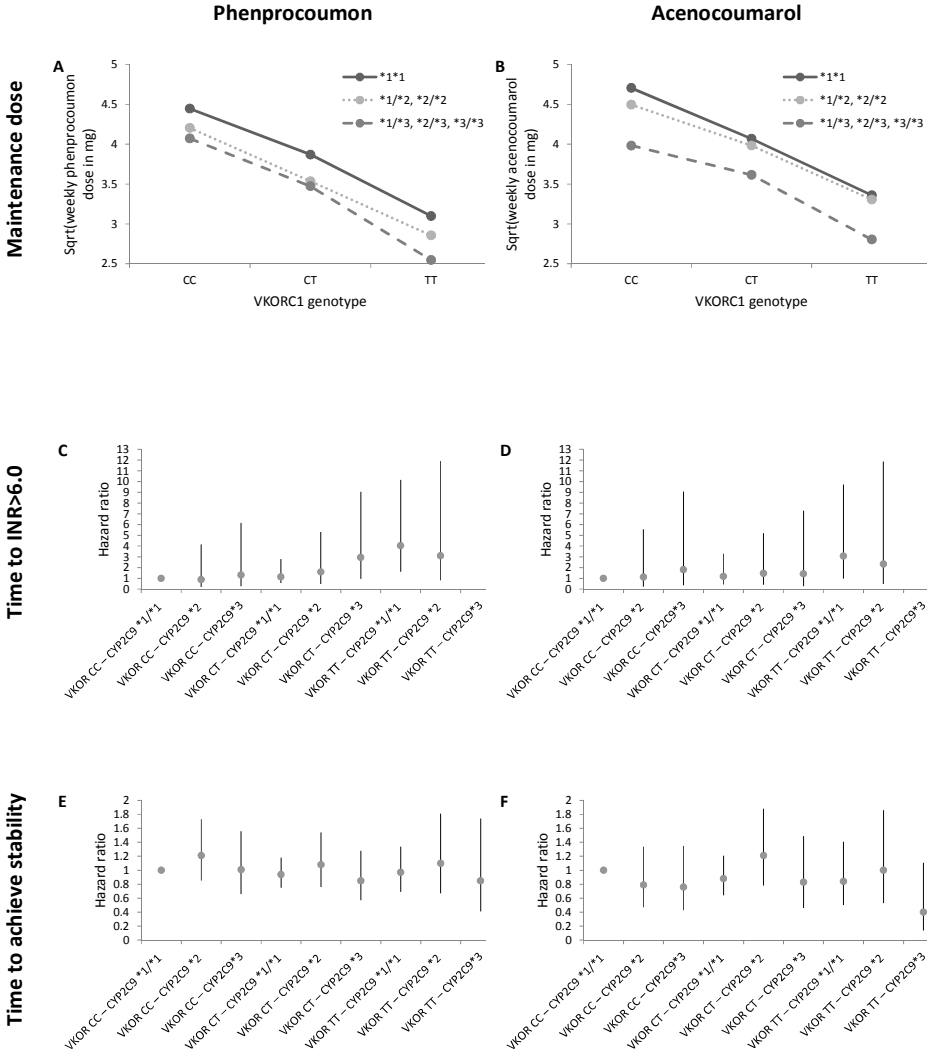

Figure 1. No gene-gene interaction between *CYP2C9* and *VKORC1* has been found for the outcome measure maintenance dose (A and B), time to severe overanticoagulation (C and D) and time to achieve stability (E and F).

Discussion

In our study, no interaction between the *CYP2C9* and *VKORC1* genotypes was present that affected the maintenance dose, time to severe overanticoagulation, or time to achieve stability of phenprocoumon and acenocoumarol. For patients carrying at least one *CYP2C9* or *VKORC1* variant allele, there was a trend for lower maintenance dosages and a trend for higher risk of overanticoagulation. No trend was seen

for stability. These results for the *CYP2C9* variant alleles were not modified by the *VKORC1* genotype and vice versa, indicating that no interaction between these two genes occurred.

Our study aimed to investigate whether a gene-gene interaction exists between the *CYP2C9* and *VKORC1* genotypes, because, to date, contradictory results have been reported[12-15]. One study did not find an interaction between the *CYP2C9* and *VKORC1* genotypes[14]. P-values were not corrected for multiple testing. However, as we did not find a significant effect, adjustment of the p-value would only have lowered the threshold and therefore would not change our outcome. Only one study on phenprocoumon discovered an interaction between the *CYP2C9* and *VKORC1* genotypes that affected the maintenance dose[13], and two acenocoumarol studies found an interaction between the genotypes, but affecting different outcome measures[12, 15]. For the outcome for which one acenocoumarol study did find an interaction, the second did not find any interaction, and vice versa. The findings of these three studies might be chance findings, because the interaction affected different outcome measures for different coumarins. Moreover, these interactions for these specific outcomes were not replicated in other studies. All studies were, like our study, performed in the Dutch population, but two studies had a smaller sample size than the population that we used for the analysis[12, 13]. Although the study of Teichert et al. had 1525 acenocoumarol users vs. 413 patients in our smallest cohort, we had enough patients in our smallest cohort to investigate a small effect with enough power. In addition, Teichert *et al*. mentioned in their discussion that the results were inconsistent, and that it is etiologically less likely that there is an interaction between the *VKORC1* and *CYP2C9* genotypes, as the mechanisms are very different.We therefore think that no interaction between the *CYP2C9* and *VKORC1* genotypes exists, and any significant effects found previously were attributable to chance findings.

Our study had some limitations. First, a selection bias might have been introduced, as we collected data from current phenprocoumon and acenocoumarol users, increasing the possibility that more long-term users were selected. However, the characteristics of our population were similar to those of other populations in other studies[10, 16, 17], suggesting that we did not introduce a selection bias. Second, it is possible that the self-reported height and weight were slightly inaccurate. Third, data on non-compliance, comedication other than amiodarone, ethnicity, smoking status and diet were not available as potential covariates for the models, as they are challenging to assess accurately and objectively. However, we assume that these parameters were equally distributed among the different classifications in this large data set. Co-medications other than amiodarone were not used as potential confounders, as they

were not significantly associated with the maintenance dose of phenprocoumon or acenocoumarol, or were too complex to define in clinical practice. Fourth, we did not perform the analysis for a more relevant clinical outcome measure, such as hemorrhages, because our data set did not provide enough data on hemorrhages.

Our findings suggest that no gene-gene interaction between the *CYP2C9* and *VKORC1* genotypes is present, which is in line with the majority of the study outcomes published on this subject. Algorithms have been developed to provide clinicians with tools when prescribing a genotype-based dose[9, 10]. As no interactions are present, these algorithms do not need adjustment.

In conclusion, we found that there is no interaction between the *CYP2C9* and *VKORC1* genotypes for the anticoagulant effect of phenprocoumon or acenocoumarol.

Supplementary material

Supplementary material is available at Journal of Thrombosis and Haemostasis online: http://onlinelibrary.wiley.com/doi/10.1111/j.1538-7836.2012.04694.x/suppinfo.

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Chapter 7

Evaluation of effects of genetic variations in *GATA-4* on the phenprocoumon and acenocoumarol maintenance dose

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Abstract

Aim

To investigate whether the phenprocoumon and acenocoumarol maintenance doses are influenced by genetic variations in *GATA-4*, a transcription factor of *CYP2C9*.

Patients & Methods

The influence of seven *GATA-4* SNPs on the coumarin maintenance dose was investigated by performing an ANOVA trend analysis, stratified for *CYP2C9* genotypes. Results of the best-explaining SNP were validated in the Rotterdam Study cohort.

Results

The largest dose differences were found for rs3735814 in patients using acenocoumarol and having the wild type allele for *CYP2C9*. The mean dosages decreased from 2.92 mg/day for the patients having the *GATA-4* wild type alleles to 2.65 mg/day for the patients carrying one *GATA-4* variant allele and to 2.37 mg/day for patients carrying two *GATA-4* variant alleles (p=0.004). Results could not be replicated in the validation cohort. For phenprocoumon, no significant effects were observed.

Conclusion

Genetic variation in *GATA-4* does not seem relevant for clinical implementation.

Introduction

Coumarin derivatives are effective medications for treating and preventing thrombosis. However, the use of coumarin is often associated with drug-related hospitalizations[1-4]. Patients receiving coumarin therapy are at risk of hemorrhage due to overdosing and to therapy failure, for example, development of a thrombus, due to underdosing[5, 6]. Coumarins have a narrow therapeutic window and therefore there is wide inter- and intra-individual variability in dose requirements[7, 8]. It is known that factors such as age, comedication, body mass, and dietary vitamin K intake influence dose requirements[9-13]. More recently, genetic factors have been shown to explain 35–50% of the inter-individual variability in dose requirements[14, 15]. In particular, polymorphisms in the *VKORC1* gene which expresses vitamin K epoxide reductase complex subunit 1 (VKORC1), the main target for coumarins, and the *CYP2C9* gene which expresses cytochrome P450 2C9 (CYP2C9), the main enzyme responsible for the metabolism of coumarins are found to influence coumarin dose requirements. Although CYP2C9 is the main metabolizing enzyme for the coumarins, genetic variation in this enzyme only explains 4.5-17.5% of the variation in maintenance dose[14, 16-22]. The contribution of *CYP2C9* to the dose variation differs per coumarin, with, in general, the lowest contribution seen for phenprocoumon.

Dose algorithms have been developed that predict the stable dose of warfarin, phenprocoumon and acenocoumarol before the start of the therapy[14, 16-22]. These algorithms explain approximately 42 to 63% of the individual dose requirements with genetic and environmental factors. Nevertheless, a large part of the dose variation remains unexplained.

Mwinyi and coworkers showed that the liver-specific transcription factor GATA-4 is involved in the transcriptional regulation of *CYP2C9*[23]. The effect of Single Nucleotide Polymorphisms (SNPs) in *GATA-4* on the CYP2C9 activity has not been investigated. It is hypothesized that genetic variation in *GATA-4* might play a role in the inter-individual variability in dose response. This study investigated whether the maintenance dose of acenocoumarol and phenprocoumon is influenced by SNPs in *GATA-4*. Since GATA-4 influences the *CYP2C9* transcription, the influence of SNPs in *GATA-4* on phenprocoumon and acenocoumarol maintenance dose could differ between *CYP2C9* genotypes. Therefore, we investigated whether the effect of *GATA-4* SNPs on the maintenance dose differs between subjects with *CYP2C9* wild type alleles and subjects with *CYP2C9* variant alleles.

Patients & Methods

Study design, patients & data collection

For the analysis, we used the Pre-EU-PACT cohort[14]. Patients currently using either phenprocoumon or acenocoumarol were eligible to take part in the study if aged 18 years and over and with a target International Normalised Ratio (INR) in the lowest intensity category (according to Dutch guidelines INR 2.0-3.5). Pregnant or breastfeeding women, patients who were in a nursing home, and patients participating in other clinical studies were excluded. Eligible patients who had a scheduled visit at the anticoagulation clinic from either 10 to 12 November 2009 (Anticoagulation Clinic Leiden, phenprocoumon) or from 23 to 27 November 2009 (Anticoagulation Clinic Medial, acenocoumarol) were invited to participate. Patients were asked to report their weight and height. Information about INRs and dosages were obtained from the electronic medical records at the anticoagulation clinics. Residual blood from INR measurements was used for genotyping. The Committee Medical Ethics Leiden approved the study protocol and procedures were in accordance with the Helsinki Declaration. A more detailed description of the study design, patients and data collection is available elsewhere[14].

Selection of the SNPs

For the selection of the SNPs, we looked at the CEU (northern and western Europe) population in Hapmap (release 28 B36). The data were further investigated and judged in Haploview (version 4.2). SNPs that were not polymorph (i.e. 100% WT) were excluded (39 SNPs). Furthermore, SNPs with a minor allele frequency (MAF) of <0.2 were removed (39 SNPs). Subsequently, all A>T (two SNPs), C>G (four SNPs), G>C (three SNPs) and T>A (one SNP) were not selected to prevent any chance of misconception of genotyping data, and therefore 38 SNPs in *GATA-4* remained.

As a final step, haploblocks were defined according to the rule of Gabriel and coworkers[24], and within each haploblock, the SNP identifying the most frequent haplotype was selected. This strategy led to six haploblocks and seven SNPs were needed to distinguish the most frequent haplotypes. Importantly, these SNPs were not in linkage disequilibrium (defined as r^2>0.7 and 'D 1). The most frequently observed haplotypes in *GATA-4* in the CEU population were identified with the following SNPs: rs12550668, rs10086064, rs3735819, rs3735814, rs2740434, rs804282 and rs904018.

Genotyping

Residual blood samples from INR measurements were used to genotype the patients for the above-mentioned SNPs using predesigned Taqman assays (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and according to manufacturers'

protocol. Common genotypes (*CYP2C9*1* and *VKORC1 C*-allele) were assigned if polymorphisms in the analyzed SNPs (*CYP2C9*2*, *CYP2C9*3*, and *VKORC1 1173C>T*) were lacking. Other variant alleles are rare in Caucasians. Therefore, there is a negligible risk for a misclassification of phenotypes due to other variant alleles. Genotypes were determined on LightCycler® 480 (Roche Diagnostics, Almere, the Netherlands) in 384-wells plates that included positive (previous established genotype) and negative controls (Tris-EDTA buffer). In addition, as quality control, 10% of the samples were genotyped in duplicate. After the genotyping results were available, the SNPs were again tested for linkage disequilibrium using PLINK.

Outcome & determinants

The outcome measure investigated was the acenocoumarol or phenprocoumon maintenance dose in the first stable period. A stable period was defined as a period of at least 3 weeks with three or more consecutive INR measurements within target range (INR 2.0-3.5) with less than 10% change in the coumarin dose. The determinants used in the analysis were the *GATA-4* SNPs.

Validation

The effect of the SNP that was most strongly related to maintenance dose requirements was validated in the Rotterdam Study cohort[25, 26]. This prospective population-based cohort study of approximately 15,000 persons was designed to investigate frequencies and determinants of different diseases, including cardiovascular diseases, in a population aged over 45 years. Complete data was available for 1,239 acenocoumarol users. The review board of The Netherlands Ministry of Health, Welfare, and Sports approved the study protocol and procedures were in accordance with the Helsinki Declaration. For phenprocoumon, the validation cohort was too small for validation (n=26). Patients were genotyped on the Illumina 550K Human Map SNP array (Illumina, San Diego, CA, USA)[27].

Statistical analysis

Analyses were stratified for *CYP2C9* genotype, namely *CYP2C9* wild type genotype (*1/*1) versus *CYP2C9* variant allele carriers. ANOVA with a linear trend test was used to compare the mean maintenance dose between *GATA-4* SNPs. P-values below 0.05 were considered significant. No correction was made for the p-values for multiple testing because the SNPs we tested were carefully selected and we validated the results in an independent population. The largest significant effect was added to the EU-PACT dose algorithm[14] in order to investigate the additional explained dose variability alongside *VKORC1* and *CYP2C9* genotype, age, height, weight, sex, and amiodarone use. SPSS version 19.0 was used in the analysis.

Results

Patient cohort

For the analyses, 571 phenprocoumon users and 398 acenocoumarol users were selected. Table 1 gives an overview of the patient characteristics.

Table 1. Characteristics of patients using phenprocoumon or acenocoumarol.

	Pre-EU-PACT cohort		Rotterdam Study cohort
	Phenprocoumon cohort (n=571)	Acenocoumarol cohort (n=398)	Acenocoumarol cohort (n=1,239)
Patient characteristics			
Age in years[a]	71.4 (44.1-88.3)	74.7 (52.7-87.6)	75.8 (60.6-89.8)
Male sex[b]	330 (57.8)	219 (55.0)	544 (43.9)
Height in cm[a]	172 (153-192)	172 (155-192)	168 (151-186)
Weight in kg[a]	80 (52-120)	79 (52-120)	75 (53-100)
Use of Amiodarone[b]	26 (4.6)	10 (2.5)	51 (4.1)
Genetic factors			
CYP2C9 genotype[b]			
*1/*1	389 (68.1)	267 (67.1)	845 (68.2)
*1/*2	103 (18.0)	76 (19.1)	241 (19.5)
*1/*3	56 (9.8)	42 (10.6)	110 (8.9)
*2/*2	10 (1.8)	7 (1.8)	23 (1.9)
*2/*3	10 (1.8)	4 (1.0)	19 (1.5)
*3/*3	3 (0.5)	2 (0.5)	1 (0.1)
VKORC1 genotype[b]			
CC	220 (38.5)	144 (36.2)	453 (36.6)
CT	267 (46.7)	204 (51.3)	595 (48.0)
TT	83 (14.5)	50 (12.6)	163 (13.2)
Missing	-	-	28 (2.3)
GATA-4 genotype (rs3735814)[b,c]			
GG	123 (21.5)	88 (22.1)	254 (20.5)
GA	268 (46.9)	203 (51.0)	564 (45.5)
AA	161 (28.2)	103 (25.9)	319 (25.7)
Missing	19 (3.3)	4 (1.0)	102 (8.2)

For the phenprocoumon users in the Pre-EU-PACT cohort, height was missing for 8 patients and weight for 3 patients. For the acenocoumarol users in the Pre-EU-PACT cohort, height was missing for 7 patients and weight for 1 patients. For the acenocoumarol users in the Rotterdam Study cohort, age was missing for 86 users, height was missing for 118 patients and weight for 116 patients.

[a] Presented is median (2.5^{th}-97.5^{th} percentile)

[b] Presented are numbers of patients (%)

[c] Genotype distributions of other SNPs are provided in Supplementary material.

GATA-4 genotyping

No inconsistencies were observed in quality control. All seven *GATA-4* SNPs were in Hardy-Weinberg equilibrium.

Effect of GATA-4 SNPs on the maintenance dose

The acenocoumarol maintenance dose increases when the number of variant alleles in SNPs in haploblock 5 increases (Figure 1). An opposite effect was observed for the SNP in haploblock 6, where, with increasing variant alleles, the dose decreases (Figure 2). This effect was no longer significant when stratified for *CYP2C9* genotype.

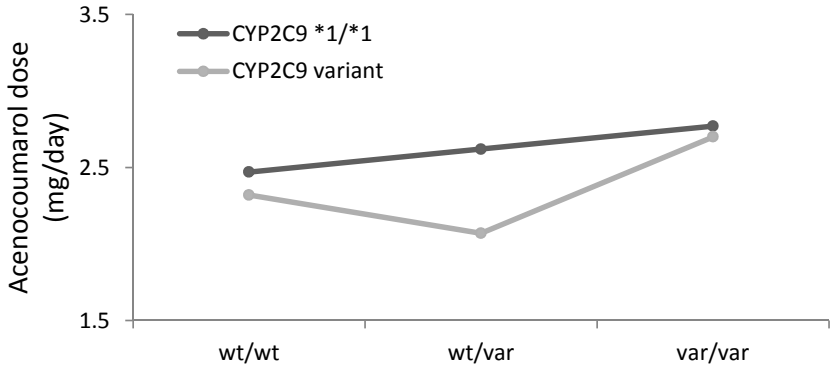

Figure 1. Effect of SNP rs804282 (located in haploblock 5, wt = wild type allele and var = variant allele) on the mean daily acenocoumarol maintenance dose. Test for trend for *CYP2C9*1/*1*: p= 0.090, for variant alleles: p= 0.160, for total: p=0.018.

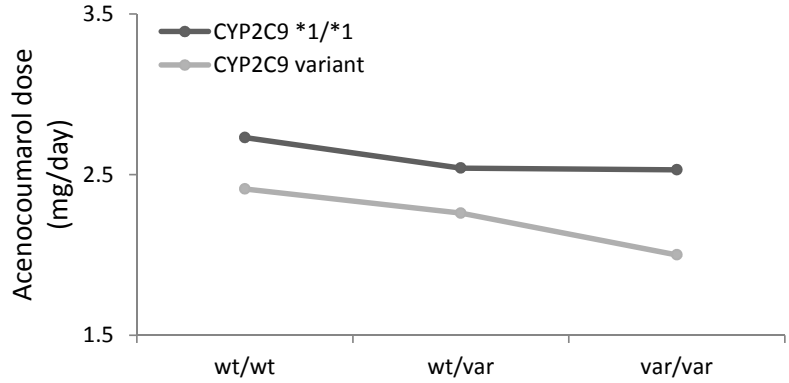

Figure 2. Effect of SNP rs904018 (located in haploblock 6, wt = wild type allele and var = variant allele) on the mean daily acenocoumarol maintenance dose. Test for trend for *CYP2C9*1/*1*: p= 0.164, for variant alleles: p= 0.114, for total: p=0.045.

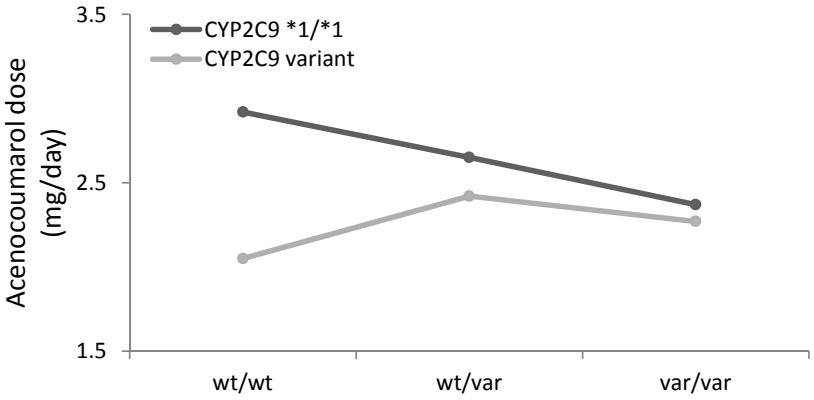

Figure 3. Effect of SNP rs3735814 (located in haploblock 4, wt = wild type allele and var = variant allele) on the mean daily acenocoumarol maintenance dose. In patients with the *CYP2C9*1/*1* genotype the acenocoumarol dose significantly decreases for patients carrying a *GATA-4* genetic variation (p=0.004).

After stratification for *CYP2C9*, genetic variations in *GATA-4* SNP rs3735814 (haploblock 4) were significantly correlated with the acenocoumarol dose in the CYP2C9*1/*1 group (p=0.004), but not in the *CYP2C9* variant allele group (Figure 3). Patients being homozygous *CYP2C9*1* require 2.92 mg/day acenocoumarol if they have the wild type genotype for rs3735814, 2.65 mg/day if they were heterozygous and 2.37 mg/day if they carried two variant alleles in rs3735814. Genetic variations in this SNP explain an additional 1.1% of the acenocoumarol dose requirements in patients with the *CYP2C9* common genotype next to *VKORC1*genotype, age, height, weight, sex, and amiodarone use (p=0.15). For patients carrying one or more *CYP2C9* variant alleles, the rs3735814 SNP only explains an additional 0.5% of the acenocoumarol dose requirements (p=0.38). For all three SNPs described above (rs804282, rs904018, and rs3735814), *VKORC1* genotypes were distributed equally across the *GATA-4* classification.

No significant relations were observed between the phenprocoumon maintenance dose and SNPs in *GATA-4*. For all data, see Supplementary material.

Validation in the Rotterdam Study

For the validation in the Rotterdam Study, 1,239 patients using acenocoumarol were available. Table 1 provides an overview of the characteristics of patients in the Rotterdam Study. In the Rotterdam Study, no effect of the *GATA-4* SNP rs3735814 was found (Figure 4). *VKORC1* genotypes were distributed equally across the *GATA-4* classification.

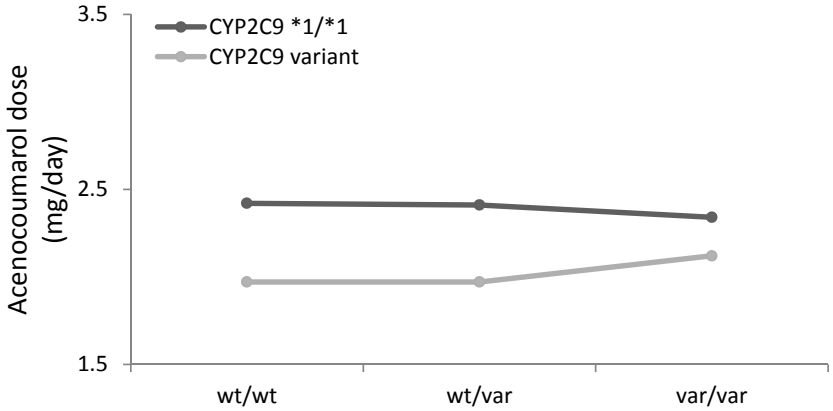

Figure 4. Effect of SNP rs3735814 (located in haploblock 4, wt = wild type allele and var = variant allele) in the Rotterdam Study cohort on the mean daily acenocoumarol maintenance dose. Test for trend for *CYP2C9*1/*1*: p= 0.370, for variant alleles: p= 0.177, for total: p=0.695).

Discussion

In this study, we found a relation between genetic variation in *GATA-4* and the acenocoumarol maintenance dose in the Pre-EU-PACT data set. The largest effect was found for SNP rs3735814 (R^2=3.2%) in *CYP2C9*1/*1* patients. Although patient height differed among the different *GATA-4* genotypes, after adjustment for height the effect of *GATA-4* on the dose was still present. However, we could not replicate these findings in the Rotterdam study. Furthermore, we did not see any association in the phenprocoumon users in the Pre-EU-PACT data set. This might be caused by the fact that, in general, CYP2C9 plays a smaller role in the metabolism of phenprocoumon compared with acenocoumarol. However, in the databases we used, the explained dose variation caused by *CYP2C9* genotypes are comparable for phenprocoumon and acenocoumarol, and were approximately 5%. This is only a small contribution to the dose variability and, therefore, it might be more difficult to find an effect of *GATA-4*. In particular if compared to other cohorts in which *CYP2C9* explain a larger proportion of the dose variation (4.5 to 17.5%)[14, 16-22]. However, since we did not find an effect of *GATA-4* on the phenprocoumon dose and were not able to replicate the found association for acenocoumarol, it is possible that the effect we found was due to chance. Our main conclusion is that the clinical implications of the seven GATA-4 SNPs we selected is of minor relevance.

To our knowledge, this is the first study that investigated the effect of genetic variation in *GATA-4* on the coumarin maintenance dose. Different dose algorithms have

been developed to estimate the warfarin[16-20], phenprocoumon and acenocoumarol maintenance dose[14, 21, 22]. Along with age, height, weight and medication use, these dose algorithms concentrate on dose variation explained by genetic variation in *VKORC1* and *CYP2C9*, together explaining up to 60% of the maintenance dose[15]. However, approximately 40% of the dose variation is still not yet explained. Different studies were performed to investigate other (genetic) covariates that could increase the explained variability. Until now, SNPs in *CYP4F2*, *CYP3A4* and *GGCX* seemed most promising, but were shown not to be as clinically relevant as *VKORC1* and *CYP2C9* polymorphisms. The (additional) dose variation explained by these genetic factors is between 0.9 and 3.3%[28-30]. In the Pre-EU-PACT study, we found that genetic variation in rs3735814 explains 3.2% of the acenocoumarol dose variation in *CYP2C9*1/*1* patients only and 0.8% for the *CYP2C9* variant allele patients, which is comparable with above-mentioned percentages. Since validation of the results was not successful, we are not convinced that *GATA-4* genotypes should be included in the dose algorithms.

This study has some limitations. We collected data from current phenprocoumon and acenocoumarol users. This approach may introduce some selection bias because longer-term users are more likely to be selected. However, the distribution of allele frequencies, amiodarone use, sex, and age is similar to other cohort studies[17, 20, 21]. Furthermore, we only genotyped seven SNPs within the *GATA-4* gene. Although these SNPs have been carefully selected, it is possible that other SNPs in *GATA-4* have a larger effect on the coumarin dose.

We hypothesized that *GATA-4* indirectly influences the coumarin maintenance dose by affecting the transcription of *CYP2C9*, and therefore, the CYP2C9 concentration. The *CYP2C9*1/*1* patients have the highest CYP2C9 metabolizing activity[31, 32], so the impact of *GATA-4* variation is expected to be largest in this group. This is in accordance with our findings. However, the effects of variations in the *GATA-4* gene, although statistically significant in some cases, seem to be small and not clinically relevant. In conclusion, genetic variation in *GATA-4* does not seem relevant for clinical implementation.

Supplementary material

Supplementary material is available at Pharmacogenomics online: http://www.futuremedicine.com/doi/suppl/10.2217/pgs.12.174.

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Chapter 8

Evaluation of the effect of SNPs in *CYP3A4* and *CYP4F2* on the stable phenprocoumon and acenocoumarol maintenance dose

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Submitted

Abstract

Aim

To investigate the effect of *CYP3A4*1B*, *CYP3A4*22* and *CYP4F2 V433M* genotypes on the acenocoumarol and phenprocoumon maintenance dose.

Patients & Methods

The Pre-EU-PACT cohort (551 phenprocoumon and 372 acenocoumarol users) was used. Linear trend analyses were performed to investigate the effect of the polymorphisms on the phenprocoumon and acenocoumarol unadjusted and adjusted maintenance dose requirements.

Results

For phenprocoumon, a significant increase in the maintenance dose of 0.13 mg/day was found for patients carrying one variant *CYP4F2* allele (n=185) if compared to wild type patients (n=325), and an even larger increase was found for patients carrying 2 *CYP4F2* variant alleles (n=41); plus 0.24 mg/day, trend-test p=0.003. For *CYP3A4*22*, a marginally significant effect was found on the phenprocoumon dose. No significant effect of *CYP3A4*1B* variant alleles on the phenprocoumon maintenance dose was found. For acenocoumarol, no significant effects on the maintenance dose were found for both *CYP4F2* and *CYP3A4* variant alleles, although the trend for *CYP4F2* was comparable to the significant trend observed for phenprocoumon.

Conclusion

The clinical relevance of these genotypes to optimize the personalized coumarin dose is low.

Introduction

Coumarin derivatives are effective medications for treating and preventing thrombosis. However, patients receiving coumarin therapy are at risk of hemorrhage due to overdosing and to therapy failure, e.g. development of a thrombus, due to underdosing[1, 2]. This is because coumarins have a narrow therapeutic window and there is wide inter- and intra-individual variability in dose requirements[3, 4], causing the use of coumarins often to be associated with drug-related hospitalizations[5-8].

It is known that factors such as age, comedication, body mass, and dietary vitamin K intake influence dose requirements[9-13]. Moreover, genetic factors have been shown to explain the largest part of the inter-individual variability in dose requirements[14, 15]. Especially polymorphisms in the *VKORC1* and *CYP2C9* gene influence coumarin dose requirements. The *VKORC1* gene expresses vitamin K epoxide reductase (VKOR), the target enzyme of coumarins. The *CYP2C9* gene expresses cytochrome P450 2C9 (CYP2C9), the main metabolizing enzyme of coumarins. The effect of polymorphisms in *CYP2C9* on the coumarin dose requirements differs per coumarin, with the lowest effect for phenprocoumon. This is because coumarins, in particular phenprocoumon, are also metabolized by other enzymes, such as CYP3A4[16, 17]. According to our knowledge, one study investigated the effect of *CYP3A4* genotypes on the coumarin dose[18]. They did not find an association between *CYP3A4*1B* genotypes and the phenprocoumon dosage. *CYP3A4*1B* is the most common variant allele, but the effects on metabolism of CYP3A4 substrates are unclear and probably of limited clinical relevance[19-21]. Wang et al.[19] recently identified a new functional SNP; designated as *CYP3A4*22*, which is associated with a decreased CYP3A4 activity and with an increased nephrotoxicity of the typical CYP3A4 substrate cyclosporine[22]. The effect of aforementioned *CYP3A4* polymorphisms on the coumarin dose requirements has not been studied yet.

Polymorphisms that have also been associated with increased coumarin dosages are variant alleles in *CYP4F2*[18, 23-28]. CYP4F2 is a vitamin K_1 oxidase. Patients carrying one or two *V433M* variant alleles in *CYP4F2* have a reduced capacity to metabolize vitamin K, resulting in increased vitamin K levels and higher coumarin dose requirements if compared to non-carriers[29]. Most studies investigated the association between the *CYP4F2* genotype and the warfarin dose[23-26]. Only two studies found an effect of *CYP4F2* genotype and the acenocoumarol dose[27, 28], and one study on the phenprocoumon dose[18].

The aim of this study was to investigate the effects of the two polymorphisms in *CYP3A4* and one polymorphism in *CYP4F2* on the stable phenprocoumon and acenocoumarol maintenance dose.

Patients & methods

Study design, patients & data collection

Patients currently using either phenprocoumon or acenocoumarol were eligible to take part in the study if aged 18 years and over and with a target INR in the lowest intensity category (according to Dutch guidelines INR 2.0-3.5). Pregnant or breast-feeding women, patients who were in a nursing home, and patients participating in other clinical studies were excluded. Eligible patients who had a scheduled visit at the anticoagulation clinic from either 10 to 12 November 2009 (Anticoagulation Clinic Leiden, phenprocoumon) or from 23 to 27 November 2009 (Anticoagulation Clinic Medial, acenocoumarol) were invited to participate. Patients were asked to report their weight and height. Information about INRs and dosages were obtained from the electronic medical records at the anticoagulation clinics. Residual INR blood was used for genotyping. The Committee Medical Ethics Leiden approved the study protocol and procedures were in accordance with the Helsinki Declaration. A more detailed description of the study design, patients and data collection can be found in our previous published article[14].

Genotyping

The SNPs in *CYP3A4* and *CYP4F2* that we selected for genotyping were rs2740574 (*CYP3A4*1B*), rs35599367 (*CYP3A4*22*) and rs2108622 (*CYP4F2 V433M*). Residual blood samples from INR measurements were used to genotype the patient for the mentioned SNPs using predesigned Taqman assays (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and according to manufacturers' protocol. Wild type genotypes were assigned if polymorphisms in the analyzed corresponding SNPs were lacking. Genotypes were determined on LightCycler® 480 (Roche Diagnostics, Almere, the Netherlands) in 384-wells plates that included positive (previous established genotype) and negative controls (TE buffer). In addition, as quality control 10% of the samples were genotyped in duplicate.

Outcome & determinants

The outcome measure investigated in this research is the phenprocoumon and acenocoumarol maintenance dose in the first stable period. A stable period is defined as a period of at least three weeks with three or more consecutive INR measurements

within target range (INR 2.0-3.5) with less than 10% change in the coumarin dose. The determinants used in the analysis are the *CYP4F2* and *CYP3A4* SNPs. We adjusted for *a priori* defined determinants that were also used in our earlier study[14], which are *CYP2C9* and *VKORC1* genotypes, age in years, sex, amiodarone use, height in cm, and weight in kg at start of the anticoagulation treatment (if this was missing current weight was used instead).

Statistical analysis

Unadjusted and adjusted mean maintenance dose differences were calculated for each genotype. Mean coumarin maintenance doses for the different genotypes were compared using linear regression with and without adjustment for possible confounders. SPSS statistics version 19.0 was used for all analyses. Results were considered significant if $p<0.05$ for the trend-test.

Results

Patient cohort

In total, 551 phenprocoumon and 372 acenocoumarol users were available for the analysis. The median age of the phenprocoumon users was 71 years and 75 years for the acenocoumarol users. More male than female patients participated in the study. Detailed patient characteristics are provided in Table 1.

Genotyping

No inconsistencies were observed for the quality controls. Allelic frequencies are summarized in Table 1. For the phenprocoumon cohort, the *CYP2C9*, *VKORC1* and *CYP3A4*22* genotypes followed Hardy-Weinberg equilibrium (HWE). Genotypes in *CYP4F2* and *CYP3A4*1B* did not follow HWE for the phenprocoumon cohort. For the acenocoumarol cohort, all genotype distributions followed HWE (Table 1).

Effects on the phenprocoumon dose

The unadjusted phenprocoumon dose differed significantly among the *CYP4F2* genotype classifications ($p=0.02$, Table 2). After adjustment for the *a priori* defined confounders, still a significant effect on the phenprocoumon dose was found ($p<0.01$, Table 2). Patients being wild type for *CYP4F2* required an adjusted phenprocoumon dose of 2.17 mg/day. This increased with 0.13 mg/day for patients carrying one variant allele and with 0.24 mg/day for patients carrying two variant alleles. In addition to the EU-PACT dose algorithms which encountered the parameters *VKORC1* and *CYP2C9* genotype, age, sex, height, weight and amiodarone use[14], *CYP4F2* genotypes

Table 1. Characteristics of patients treated with phenprocoumon and acenocoumarol.

	Phenprocoumon cohort (n=551)	Acenocoumarol cohort (n=372)
Patient characteristics		
Median age in years (2.5th -97.5th percentile)	71.1 (44.4-88.4)	74.8 (52.3-87.5)
Male sex (%)	323 (58.6)	210 (56.5)
Median height in cm (2.5th -97.5th percentile)	172 (153-192)	172 (154-193)
Median weight in kg (2.5th -97.5th percentile)	80 (52-120)	79 (52-120)
Use of Amiodarone (%)	26 (4.7)	9 (2.4)
Genetic factors		
CYP2C9 genotype (n,(%))[a]		
*1/*1	375 (68.1)	249 (66.9)
*1/*2	101 (18.3)	71 (19.1)
*1/*3	53 (9.6)	39 (10.5)
*2/*2	10 (1.8)	7 (1.9)
*2/*3	9 (1.6)	4 (1.1)
*3/*3	3 (0.5)	2 (0.5)
Allelic frequency	*1=82%, *2=12%, *3=6%	*1=82%, *2=12%, *3=6%
VKORC1 genotype (n, (%))[b]		
CC	215 (39.0)	137 (36.8)
CT	254 (46.1)	188 (50.5)
TT	82 (14.9)	47 (12.6)
Allelic frequency	C=64%, T=36%	C=62%, T=38%
CYP4F2 genotype (n, (%))[c]		
CC	325 (59.0)	196 (52.7)
CT	185 (33.6)	143 (38.4)
TT	41 (7.4)	33 (8.9)
Allelic frequency	C=76%, T=24%	C=72%, T=28%
*CYP3A4*1B* genotype (n, (%))[d]		
AA	504 (91.5)	342 (91.9)
AG	43 (7.8)	30 (8.1)
GG	4 (0.7)	-
Allelic frequency	A=95%, G=5%	A=96%, G=4%
*CYP3A4*22* genotype (n, (%))[e]		
CC	496 (90.0)	323 (86.8)
CT	54 (9.8)	47 (12.6)
TT	1 (0.2)	2 (0.5)
Allelic frequency	C=95%, T=5%	C=93%, T=7%

[a] HWE: phenprocoumon χ^2= 1.70, p= 0.64, acenocoumarol χ^2= 1.23, p= 0.75.
[b] HWE: phenprocoumon χ^2= 0.24, p= 0.62, acenocoumarol χ^2= 2.01, p= 0.16.
[c] HWE: phenprocoumon χ^2= 4.03, p= 0.04, acenocoumarol χ^2= 0.88, p= 0.35.
[d] HWE: phenprocoumon χ^2= 7.41, p= 0.006, acenocoumarol χ^2= 0.66, p= 0.42.
[e] HWE: phenprocoumon χ^2= 0.14, p= 0.71, acenocoumarol χ^2= 0.04, p= 0.84.

Table 2. Unadjusted and adjusted mean phenprocoumon maintenance dose per *CYP4F2*, *CYP3A4*1B* and *CYP3A4*22* genotype.

	Genotype	n	Unadjusted difference mean (95% CI)[a]	Adjusted[c] difference mean (95% CI)[b]
CYP4F2	Homozygous wild type (CC)	325	Reference: 2.16 mg/day	Reference: 2.17 mg/day
	Heterozygous (CT)	185	+0.13 (-0.03 to +0.29)	+0.13 (+0.02 to +0.24)[‡]
	Homozygous variant (TT)	41	+0.31 (+0.02 to +0.59)	+0.24 (+0.04 to +0.44)[‡]
*CYP3A4*1B*	Homozygous wild type (AA)	504	Reference: 2.23 mg/day	Reference: 2.23 mg/day
	Heterozygous (AG)	43	+0.02 (-0.26 to +0.29)	+0.06 (-0.14 to +0.25)
	Homozygous variant (GG)	4	+0.18 (-0.70 to +1.05)	-0.47 (-1.17 to +0.05)
*CYP3A4*22*	Homozygous wild type (CC)	496	Reference: 2.26 mg/day	Reference: 2.25 mg/day
	Heterozygous (CT)	54	-0.25 (-0.50 to -0.00)	-0.18 (-0.35 to -0.01)
	Homozygous variant (TT)	1	-0.61	+0.12

[a] Trend-test *CYP4F2*: p= 0.02, *CYP3A4*1B*: p= 0.77, *CYP3A4*22*: p= 0.04
[b] Trend-test *CYP4F2*: p< 0.01, *CYP3A4*1B*: p= 0.65, *CYP3A4*22*: p= 0.05
[c] Adjusted for *CYP2C9* and *VKORC1* genotype, age, sex, height, weight, and amiodarone use.
[‡]Significant difference if compared to reference group.

further explained 0.8% of the square root dose variation. The percentage dose variation explained by only *CYP4F2* genotypes was 1.1%.

No effect of *CYP3A4*1B* genotypes on the unadjusted (p=0.92) and adjusted (p=0.16) phenprocoumon dose have been found (Table 2).

Marginally significant effects were observed for the association between *CYP3A4*22* genotypes and the unadjusted and adjusted phenprocoumon dose, p=0.04 and p=0.05, respectively (Table 2). Variant alleles were negatively related with the stable phenprocoumon dose.

Effects on the acenocoumarol dose

Although a similar trend was observed for *CYP4F2* variant alleles on the acenocoumarol dose as was observed for phenprocoumon, *CYP4F2* genotypes did not significantly affect the unadjusted and adjusted acenocoumarol maintenance dose (p=0.11 and p=0.09, respectively, Table 3). In addition, no effect was found for *CYP3A4*1B* (p=0.40 and p=0.39 for the unadjusted and adjusted maintenance dose, respectively, Table 3) and for *CYP3A4*22* (p=0.91 and p=0.57 for the unadjusted and adjusted maintenance dose, respectively, Table 3).

Table 3. Unadjusted and adjusted mean acenocoumarol maintenance dose per *CYP4F2*, *CYP3A4*1B* and *CYP3A4*22* genotype.

	Genotype	n	Unadjusted difference mean (95% CI)[a]	Adjusted[c] difference mean (95% CI)[b]
CYP4F2	Homozygous wild type (CC)	196	Reference: 2.48 mg/day	Reference: 2.46 mg/day
	Heterozygous (CT)	143	+0.02 (-0.20 to +0.24)	+0.11 (-0.05 to +0.27)
	Homozygous variant (TT)	33	+0.40 (+0.03 to +0.77)	+0.24 (-0.04 to +0.52)
*CYP3A4*1B*	Homozygous wild type (AA)	342	Reference: 2.54 mg/day	Reference: 2.53 mg/day
	Heterozygous (AG)	30	-0.16 (-0.54 to +0.21)	-0.12 (-0.40 to +0.15)
	Homozygous variant (GG)	0	-	-
*CYP3A4*22*	Homozygous wild type (CC)	323	Reference: 2.53 mg/day	Reference: 2.52 mg/day
	Heterozygous (CT)	47	-0.07 (-0.38 to +0.24)	+0.03 (-0.20 to +0.26)
	Homozygous variant (TT)	2	+0.56 (-0.85 to +1.96)	+0.40 (-0.66 to +1.46)

[a] Trend-test *CYP4F2*: p= 0.11, *CYP3A4*1B*: p=0.40, *CYP3A4*22*: p=0.91
[b] Trend-test *CYP4F2*: p= 0.09, *CYP3A4*1B*: p= 0.39, *CYP3A4*22*: p= 0.57
[c] Adjusted for *CYP2C9* and *VKORC1* genotype, age, sex, height, weight, and amiodarone use.

Discussion

In this study we found that polymorphisms in *CYP4F2* are associated with an increase in the phenprocoumon and acenocoumarol maintenance doses, although these effects were only statistically significant for phenprocoumon. However, the observed effects were small and probably not clinically relevant. Polymorphisms in *CYP3A4*22* affect the phenprocoumon but not the acenocoumarol dose requirements. No association between *CYP3A4*1B* genotypes and both the phenprocoumon and acenocoumarol maintenance dose have been found.

These findings -only a small effect of genetic variations in *CYP4F2* on the coumarin (warfarin, acenocoumarol and phenprocoumon) maintenance dosages- are in line with previous studies[18, 23-28]. Although we did not find a significant effect for *CYP4F2* polymorphisms on the acenocoumarol dose, the trend of increasing dose requirements with increasing variant alleles is in line with earlier findings. However, adding *CYP4F2* to the phenprocoumon and acenocoumarol dose calculators[14] to further optimize personalized dosing does not seem relevant, since addition of this polymorphism only explains an extra 0.8% of the dose variation. This is also in line with the previous studies. The reason that dose requirements are increased for patients carrying one or 2 *CYP4F2* alleles, was explained by McDonald et al.[29]; patients having polymorphisms in *CYP4F2* have lowered capacity to metabolize vitamin K. This increases the vitamin K levels and therefore increases the coumarin dose requirements.

This is the first study that investigated the effect of *CYP3A4*22* on the coumarin maintenance dose. The effect of *CYP3A4*1B* on the phenprocoumon dose has been investigated before[18] and that study did not find an effect on the phenprocoumon maintenance dose. Also in our study no association between the coumarin maintenance dose and *CYP3A4* polymorphisms was found, neither for acenocoumarol nor for phenprocoumon. That we did not find an association between the *CYP3A4* genotype and acenocoumarol dose requirements, is not surprising since *CYP3A4* is not involved in the metabolism of neither S-acenocoumarol nor R-acenocoumarol[16]. However, CYP3A4 does play a significant role in the metabolism of both the more active S- enantiomer as well as the R-enantiomer of phenprocoumon[16, 17]. The role of the *CYP3A4*1B* polymorphism in CYP3A4 activity is not clear. A meta-analysis of the pharmacokinetic parameters of 7 clincial trials in which the CYP3A4 monosubstrate midazolam was administered, reported no association between the *CYP3A4*1B* SNP and midazolam disposition in vivo[21]. This apparent lack of effect of *CYP3A4*1B* on the pharmacokinetics of a CYP3A4 monosubstrate is in agreement with a lack of effect of this polymorphism on dose requirements of phenprocoumon, in which CYP3A4 plays a far more limited metabolizing role than in midazolam. The explanation for the apparent lack of an association between the *CYP3A4*22* SNP and phenprocoumon dose requirements is more difficult to explain, since being carrier of this SNP has clearly been associated with a decreased CYP3A4 activity and an increased risk of adverse effects of the CYP3A4 substrate cyclosporin[22]. The only explanation is that the impact of this polymorphism is limited for a substance such as phenprocoumon in which CYP3A4 represents a secondary metabolic pathway.

In our study, *CYP4F2* and *CYP3A4*1B* were not in HWE for phenprocoumon. No inconsistencies were found with the controls during the genotyping process. In addition, whether a genotype is in HWE or not does not affect the prognostic value. It might provide information on that a certain population was selected. We therefore assume that this does not affect our results.

In conclusion, genetic variations in *CYP4F2* appear to marginally increase the stable phenprocoumon maintenance dose. The same trend, although not significant, is found for acenocoumarol. No statistically significant effect is observed for *CYP3A4* genotypes for both phenprocoumon and acenocoumarol. The clinical relevance of these genotypes to optimize the personalized coumarin dose is low.

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Chapter 9

Evaluation of the effect of statin use on the acenocoumarol and phenprocoumon maintenance dose

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Abstract

Aim

Statins and coumarins are prescribed in combination on a regular basis. Some case reports suggested that statins might affect the dose requirements of coumarins. The aim of the study was to investigate whether acenocoumarol and phenprocoumon maintenance doses are influenced by statin use.

Patients & methods

The Pre-EU-PACT database was used, which contains information on 471 acenocoumarol and 624 phenprocoumon users. The influence of individual statins on the acenocoumarol and phenprocoumon maintenance dose was investigated by comparing unadjusted and adjusted mean differences of the maintenance dose between statin and non-statin users.

Results

Lower adjusted acenocoumarol dose requirements were observed for patients using atorvastatin, simvastatin, pravastatin, and rosuvastatin. These patients had a reduction in adjusted mean acenocoumarol maintenance dose of 0.11, 0.29, 0.38, and 0.69 mg/day, respectively, compared with a mean adjusted dose of 2.60 mg/day for the patients not using a statin. There was no significant effect of statin use on unadjusted and adjusted phenprocoumon maintenance dose ($p=0.23$ and $p=0.35$, respectively).

Conclusion

Mean acenocoumarol maintenance dosages were decreased when acenocoumarol was coadministered with the different statins. Statin use did not affect phenprocoumon maintenance doses significantly.

Introduction

In the Netherlands, almost 1.7 million people, which is over 10 % of the total Dutch population, used a statin in 2010 and over 376,000 (2%) used oral anticoagulants[1]. Hence, simultaneous use will occur often, particularly because both drugs have joint indications (cardiovascular disease). The statins prescribed in The Netherlands are simvastatin (55.4%), atorvastatin (22.4%), rosuvastatin (10.8%), pravastatin (10.1%), and fluvastatin (1.3%)[1]. The most prescribed oral anticoagulant in the Netherlands is the vitamin K antagonist acenocoumarol, which has over 300,000 users. To a lower extent, phenprocoumon is prescribed (76,000 patients)[1].

Many different drug interactions have been described for acenocoumarol and phenprocoumon[2], but the interaction between statins and acenocoumarol and phenprocoumon has, to our knowledge, not been investigated. Several case reports have been published of altered therapy control of acenocoumarol and warfarin during the use of a statin[3-8]. Also, some small studies have investigated the effect of statin use on warfarin therapy[9-12]. These studies showed that warfarin doses are decreased if coadministered with either rosuvastatin or simvastatin and that the pravastatin could be safely changed to simvastatin. Furthermore, rosuvastatin has been placed on a Dutch list of coumarin interactions because concomitant use of these drugs was found to result frequently in decreased coumarin dose requirements[13]. However, evidence for this special position for rosuvastatin in comparison to other statins and the effects of different statins on acenocoumarol and phenprocoumon are lacking. Therefore, we investigated the effects of coadministration of statins on the acenocoumarol or phenprocoumon maintenance dose.

Patients & methods

Study design, patient selection, & data collection

For this study, the Pre-EU-PACT data set was used[14]. Patients currently using acenocoumarol or phenprocoumon were invited to participate if they had a scheduled visit at the anticoagulation clinic from either 23 to 27 November 2009 (acenocoumarol; Anticoagulation Clinic Medial) or from 10 to 12 November 2009 (phenprocoumon; Anticoagulation Clinic Leiden). They were eligible to take part in the study if aged 18 years and older. Pregnant or breastfeeding women, patients who were in a nursing home, and patients participating in other clinical studies were excluded. Patients were asked to report their weight and height. Information about International Normalized Ratios (INRs), dosages, and comedication were obtained from the electronic

medical records at the anticoagulation clinics. The prescribed dose of comedication is not routinely registered by the anticoagulation clinic. The Committee Medical Ethics Leiden approved the study protocol, and procedures were in accordance with the Helsinki Declaration. A more detailed description of the study design, patients, and data collection is available[14].

Patients were selected when they had a therapeutic INR range between 2.0 and 3.5 and a stable coumarin maintenance dose. This was defined as the prescribed dose in the first stable period, which was a period of at least 3 weeks and with three or more consecutive INR measurements within the therapeutic range with less than 10% change in the coumarin dose. In addition, patients were excluded if they needed more than 365 days to reach stable dose or if they had incomplete data for *VKORC1* (gene corresponding for vitamin K epoxide reductase complex subunit 1, the target enzyme of coumarins) and *CYP2C9* (gene corresponding for cytochrome P450 (CYP) 2C9, the main metabolizing enzyme of coumarins) genotype, height, or weight. Residual venous blood was used for genotyping *VKORC1 1173C>T* (rs9934438), *CYP2C9*2* (rs1799853), and *CYP2C9*3* (rs1057910).

Outcome measure & determinants

The outcome measure investigated was the acenocoumarol or phenprocoumon maintenance dose in the first stable period. Information from the records of the anticoagulation clinic, which monitors comedication use but not dosages, was used to establish statin use.

Statistical analysis

Analyses were carried out separately for acenocoumarol and phenprocoumon. Between-group p-values were determined for dose differences between the individual statins and no-statin use. If significant dose differences were present, mean maintenance doses were compared between users of individual statins and non-statin users using linear regression, with and without adjustment for *a priori* defined possible confounders. Adjustments were made for *VKORC1* and *CYP2C9* genotype, age, weight, height, sex, and amiodarone use. Sensitivity analyses were performed excluding patients using the CYP2C9 inhibitor amiodarone. Dose differences, with 95% confidence intervals (CIs), were calculated if a significant effect on the coumarin dose was found. SPSS statistics, version 19.0 (SPSS, Chicago, IL, USA), was used for all analyses.

Results

Patient cohort

For this study, 375 acenocoumarol and 559 phenprocoumon users were selected. There were 98 acenocoumarol patients (26.1 %) and 152 phenprocoumon patients (27.2 %) who used a statin. Statin users used more often amiodarone. All genotypes were in Hardy-Weinberg equilibrium (HWE), except the *VKORC1* genotype for the statin-users group within the acenocoumarol users (p = 0.04). The characteristics of the patients are given in Table 1.

Table 1. Characteristics of patients treated with acenocoumarol and phenprocoumon.

	Acenocoumarol cohort (n=375)		Phenprocoumon cohort (n=559)	
Patient characteristics	**Non-statin users (n=277)**	**Statin users (n=98)**	**Non-statin users (n=407)**	**Statin users (n=152)**
Age in years[a]	75 (47-88)	75 (57-87)	71 (43-89)	71 (50-87)
Male sex[b]	163 (58.8)	49 (50.0)	235 (57.7)	91 (59.9)
Height in cm[a]	173 (155-197)	170 (153-190)	172 (153-192)	174 (154-190)
Weight in kg[b]	79 (50-125)	90 (56-114)	79 (52-120)	80 (55-118)
Use of Amiodarone[b]	5 (1.8)	4 (4.1)	13 (3.2)	13 (8.6)
Use of Statin[b]	-	98	-	152
• Atorvastatin	-	20 (20.4)	-	38 (25)
• Simvastatin	-	55 (56.1)	-	54 (35.5)
• Pravastatin	-	18 (18.4)	-	50 (32.9)
• Rosuvastatin	-	5 (5.1)	-	9 (5.9)
• Fluvastatin	-	0 (0)	-	1 (0.7)
Genetic factors				
CYP2C9 genotype[b,c]				
*1/*1	183 (66.1)	69 (70.4)	290 (71.3)	91 (59.9)
*1/*2	59 (21.3)	12 (12.2)	73 (17.9)	29 (19.1)
*1/*3	27 (9.7)	12 (12.2)	33 (8.1)	21 (13.8)
*2/*2	5 (1.8)	2 (2.0)	6 (1.5)	4 (2.6)
*2/*3	1 (0.4)	3 (3.1)	3 (0.7)	6 (3.9)
*3/*3	2 (0.7)	-	2 (0.5)	1 (0.7)
VKORC1 genotype[b,d]				
CC	106 (38.3)	32 (32.7)	166 (40.8)	52 (34.2)
CT	134 (48.4)	56 (57.1)	182 (44.7)	77 (50.7)
TT	37 (13.4)	10 (10.2)	59 (14.5)	23 (15.1)

[a] Presented is median (2.5^{th}-97.5^{th} percentile)
[b] Presented are numbers of patients (%)
[c] HWE, statin and non-statin users together: acencoumarol χ^2=1.25, p=0.54, phenprocoumon χ^2=1.62, p=0.44.
[d] HWE, statin and non-statin users together: acencoumarol χ^2=2.21, p=0.14, phenprocoumon χ^2=0.13, p=0.72.

Effects of different statins on the acenocoumarol dose

The between-group p-value was 0.003 for the unadjusted acenocoumarol dose and 0.008 for the adjusted acenocoumarol dose. Therefore, it is allowed to compare the effects on the acenocoumarol maintenance dose for each separate statin to the reference group that did not use a statin. Patients using simvastatin, pravastatin, or rosuvastatin had a significantly lower unadjusted maintenance dose than non-statin users, whereas the maintenance dose was higher, although not significantly, when atorvastatin was used. The adjusted acenocoumarol doses were lower for all statins, but not significantly for atorvastatin (Table 2). Effects did not change when patients using amiodarone were excluded.

Table 2. Unadjusted and adjusted mean acenocoumarol maintenance dose per (non-) statin group.

	n	Unadjusted mean difference (95% CI)[b]	Percentage unadjusted mean difference	Adjusted[a] mean difference (95% CI)[b]	Percentage adjusted mean difference
No statin use	277	Reference: 2.61 mg/day	Reference:100	Reference: 2.60 mg/day	Reference: 100
Atorvastatin	20	+0.16 (-0.29 to +0.61)	106.1 (88.9-123.4)	-0.11 (-0.45 to +0.22)	95.4 (82.7-108.0)
Simvastatin	55	-0.37 (-0.66 to -0.08)[‡]	85.8 (74.7-103.1)	-0.29 (-0.51 to -0.08)[‡]	88.8 (80.4- 96.9)
Pravastatin	18	-0.51 (-0.98 to -0.04)[‡]	80.5 (62.5- 98.5)	-0.38 (-0.73 to -0.02)[‡]	85.3 (71.9-99.2)
Rosuvastatin	5	-1.09 (-1.96 to -0.21)[‡]	58.2 (24.9-92.0)	-0.69 (-1.35 to -0.03)[‡]	73.5 (48.1-98.8)

[a] Adjusted for *CYP2C9* and *VKORC1* genotype, age, sex, height, weight, and amiodarone use
[b] Between group p-value <0.01, therefore conclusions can be drawn from CIs for separate statins
[‡] $p < 0.05$, compared to no statin use

Effects of different statins on the phenprocoumon dose

The unadjusted and adjusted mean phenprocoumon dose did not significantly differ between patient groups using atorvastatin, simvastatin, pravastatin, rosuvastatin,

Table 3. Unadjusted and adjusted mean phenprocoumon maintenance dose per (non-) statin group.

	n	Unadjusted mean difference (95% CI)	Percentage unadjusted mean difference	Adjusted[a] mean difference (95% CI)	Percentage adjusted mean difference
No statin use	407	Reference: 2.28 mg/day	Reference: 100	Reference: 2.24 mg/day	Reference: 100
Atorvastatin	38	-0.09	96.1	-0.01	99.6
Simvastatin	54	-0.19	91.7	+0.04	101.8
Pravastatin	50	-0.28	87.7	-0.20	91.1
Rosuvastatin	9	+0.20	108.8	+0.15	106.7
Fluvastatin	1	-0.43	81.1	+0.17	107.6

[a] Adjusted for *CYP2C9* and *VKORC1* genotype, age, sex, height, weight, and amiodarone use

fluvastatin, or no statin (p=0.23 and p=0.35, respectively; Table 3). Effects did not change when patients using amiodarone were excluded.

Discussion

We found that the acenocoumarol maintenance dosages were significantly decreased when acenocoumarol was coadministered with simvastatin, pravastatin, or rosuvastatin. For phenprocoumon, no significant effects of statin use on the maintenance dose were found.

As far as we know, this is the first study investigating the effect of statin use in patients taking acenocoumarol or phenprocoumon. The findings of three small studies for warfarin are in line with our results: an enhanced effect of warfarin when co-administered with rosuvastatin[9] and simvastatin[10, 11]. In addition, case reports described the enhanced anticoagulation effect of statin use of rosuvastatin and simvastatin on the acenocoumarol therapy[3, 4]. Furthermore, Westergren *et al*.[6] reported that when atorvastatin (which does not affect warfarin dose requirements) was changed to simvastatin (which requires warfarin dose reduction), there were enhanced anticoagulation effects. Unfortunately, in our study, only one patient used fluvastatin, so we could not conclude on any effect of fluvastatin use on coumarin dose requirements and compare it with the literature[5, 8].

The mechanism behind the observed effects on the acenocoumarol dose by statins is unclear. Lennernas *et al*. hypothesized displacement of coumarins from plasma albumin when co-administered with a statin[15]. However, drug interactions based on albumin displacement are of short duration and rarely clinically relevant[16]. A uniform pharmacokinetic mechanism, if any, is difficult to hypothesize. It is doubtful whether an interaction effect can be explained by CYP inhibition. Acenocoumarol is mainly metabolized by CYP2C9[2]. Rosuvastatin, the statin with the strongest effect on the acenocoumarol dose requirement in our study, does not affect CYP isoenzymes, and CYP isoenzymes do not play an important role in its metabolism either[17]. Pravastatin is mainly metabolized by other pathways than the CYP enzymes and does not affect cytochrome isoenzymes[18]. Simvastatin and atorvastatin are both metabolized by CYP3A4[15, 19, 20], but in our study, only simvastatin had a significant effect on acenocoumarol dose requirements. Moreover, dose requirements of phenprocoumon, which is at least partly metabolized by CYP3A4[2], were not significantly affected by any statin. Furthermore, it is possible that the observed effect of statin use on the acenocoumarol maintenance dose is caused by underlying reasons for

the use of statins. Further research is required to provide insight in the biological mechanisms.

The main limitation of this study is that the information on dosages was missing and that co-medication use was reported at the anticoagulation clinic. It is possible that there are discrepancies between the medication records of the anticoagulation clinics and the pharmacy records[21]. However, the anticoagulation clinics checked co-medication use at the start of the coumarin therapy. In our database, all statin users used a statin at the time that a coumarin was prescribed. No stopping, switching, or initiation of statin use was reported during coumarin therapy for any of the patients. We therefore assumed that comedication information was complete and that no changes have been made regarding the statin therapy during the anticoagulant therapy. The frequency of statin use was similar in the two anticoagulation clinics in our study.

The *VKORC1* genotypes were not in HWE for statin users within the group of patients using acenocoumarol. As no equilibrium was found only in this subgroup, and not significant after adjustment for multiple testing, we assume that this was due to chance and anticipate no effect on the measured outcomes.

The results of our study indicate that physicians should take into account the effect of statin use on the anticoagulant therapy with acenocoumarol. Effects on the phenprocoumon maintenance dose seem clinically irrelevant. Our results also suggest that concomitant use of atorvastatin could be less problematic in acenocoumarol users than concomitant use of other statins. In practice, the unadjusted dose differences would be of more interest than the adjusted dose differences, as *CYP2C9* and *VKORC1* genotypes – which are taken into account when calculating the adjusted dose difference – are often unknown. At the start of anticoagulant therapy, dose differences caused by statin use are too small to take into account. The start of co-administration of any statin during the anticoagulant therapy is anticipated to cause a small dose adjustment of the coumarin, which is why we recommend that patients should be more frequently monitored.

In conclusion, we found lower maintenance doses when acenocoumarol was co-administered with statins, in particular, for simvastatin, pravastatin, or rosuvastatin. No significant effects of statin use on the phenprocoumon maintenance dose were observed. Physicians should be aware that statins can lower the coumarin maintenance dose, especially in combination with acenocoumarol.

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Chapter 10

General discussion

Part of this chapter is based on:

Future of pharmacogenetics in cardiovascular diseases

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Scope of this thesis

Several gene-drug interactions have been discovered in the field of cardiovascular diseases (CVDs). These gene-drug interactions can help to identify a decreased or increased response to drugs, estimate dose requirements or identify an increased risk of developing adverse drug reactions. An individualized approach based on pharmacogenetic testing will provide physicians and pharmacists with tools for decision making about pharmacotherapy. While pharmacogenetic testing is already part of everyday practice in for example oncology, it is not yet implemented in the field of CVDs.

Cardiovascular drugs are widely used for prevention and treatment of CVD. Prophylaxis and treatment of CVD is complex. Patients often have more than one cardiovascular risk factor (e.g. hypertension and hypercholesterolemia) and CVD, or other comorbidities such as diabetes. Frequently, more than one drug is used by the patient and this may potentially lead to serious drug interactions with adverse health outcomes. Therefore, not only the morbidities but also the interaction between medications should be taken into account if a pharmacogenetics based dosing strategy is developed.

Gene-drug interactions were demonstrated for antihypertensive drugs[1], statins[2, 3], platelet inhibitors[4], and anticoagulants[5, 6]. The findings of the many studies that have been conducted on pharmacogenetics are up to now not suitable for clinical implementation, often because the results could not be replicated or the clinical relevance is low. However, especially coumarins might be candidate for pharmacogenetic testing in everyday practice.

This thesis discusses the pharmacogenetics of the coumarins. Oral anticoagulants of the coumarin type (phenprocoumon, acenocoumarol and warfarin being the most important ones) are used to treat and prevent thromboembolic events in patients with different conditions, the most common indications being venous thromboembolism and atrial fibrillation[7]. The effect of coumarins is monitored by determining the International Normalized Ratio (INR), which should be kept within a certain range (for example, the range for atrial fibrillation is between 2.0 and 3.0 in most countries). Coumarins have a wide inter- and intra-patient variability in dose requirement which means that the dosage is difficult to predict and frequent monitoring of the INR is necessary. INR values below the therapeutic range increase the risk of thromboembolic events while a supra-therapeutic INR leads to an increased risk of hemorrhagic events. These hemorrhages can range from minor to major, life-threatening and fatal hemorrhages such as an intracranial hemorrhage[8].

The wide variability in dose requirement is caused by several factors. Dietary intake of vitamin K, comorbidities (e.g. altered thyroid function), concomitant medication (e.g. amiodarone), sex, age, height and weight all influence the required coumarin dose[9-15]. Also genetic factors have been shown to play an important role[16-18]. First the influence of variation within the *CYP2C*9 gene, encoding the main metabolizing enzyme, cytochrome P450 2C9 (CYP2C9) was discovered. Carriers of a *2 or *3 allele require a lower dose and have an increased risk of overanticoagulation, which is associated with an increased risk of hemorrhage[19]. A few years later it was discovered that variation within the *VKORC1* gene, encoding the target enzyme vitamin K epoxide reductase multiprotein complex 1, explains an even larger part of dose requirement variability. *CYP2C9* and *VKORC1* together explain up to half of the variation in coumarin dose requirement[5, 9, 20]. Other factors like age, height, weight, sex, and concomitant amiodarone use add another 10 to 20% explanation of variation in response[9, 10].

Currently, most patients receive an identical initial coumarin dosage. After a few days, the response to this initial dose is evaluated by INR measurement. Subsequently, the dose is adapted according to the patient's needs to achieve an adequate INR. If patients are genotyped before starting coumarin therapy, they can receive a genotype-guided dose from day 1 on. This may prevent overanticoagulation in carriers of a variant allele and may enable patients to reach a stable dose earlier. During this PhD project, we have included patients in a randomized controlled trial (EU-PACT) to be able to provide evidence for the (cost) effectiveness of pretreatment genotyping for coumarin derivatives[21].

In this discussion we will elaborate on the results described in this thesis. Firstly, we will discuss our main findings and place them in a broader perspective. Secondly, we will argue how to implement pharmacogenetics and which parties are involved in that; we will discuss the use of clinical trials in pharmacogenetics to provide evidence, we will elaborate on the necessary facilities to implement pharmacogenetics, we will consider whether implementing genetic testing will be cost-effective, and discuss technical developments in pharmacogenetics. Thirdly, recent developments in oral anticoagulant therapy will be discussed, and the implications of these developments on the work in this thesis. Finally, we will provide suggestions for future research.

Main Findings

Development and usage of the phenprocoumon and acenocoumarol dose algorithms

In a large patient population we developed and validated the first phenprocoumon and acenocoumarol loading and maintenance dose algorithms to be used in clinical practice[9] (Chapter 3 and 4). We included genetic data (variation in *CYP2C9* and *VKORC1*) and patient characteristics such as age and weight in the algorithms. The dose algorithms explain up to 56% of the variability in maintenance dose, which is comparable with earlier developed warfarin dose algorithms in which also genotype information of *CYP2C9* and *VKORC1* were used[10, 22-25]. When only patient characteristics, i.e. age, height, weight, sex and amiodarone use, are taken into account, these algorithms explain 17% of the phenprocoumon dose variability and 24% of acenocoumarol dose variability. We validated our results in independent data sets[26-28], which showed that the algorithms were also adequately predicting the maintenance dose in other populations.

Our two aforementioned studies were both performed retrospectively. In order to draw conclusions on the clinical applicability of the dose algorithms and whether they improve oral anticoagulant therapy in terms of increased safety and efficacy, they need to be tested prospectively in a randomized controlled trial. Our dosing algorithms are currently being tested in the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial (Chapter 5)[21]. This is one of the larger trials investigating pharmacogenetic guided dosing (algorithms with genotype information versus algoritms without genotype information or standard clinical care) of coumarins. EU-PACT is the only trial that focuses on 3 coumarin derivatives; warfarin, which is mainly used in the US, UK and Sweden, and phenprocoumon and acenocoumarol, the coumarins being mainly used in continental Europe. Results are expected to become available in mid 2013.

Effects of genetic variance and comedication use on anticoagulant therapy

Although we are able to explain almost 60% of the dose variability, still more than 40% is unknown. Therefore we performed studies to further investigate dose variation. These studies are described in Part III of this thesis: "Effects of genetic variance and comedication use on the anticoagulant therapy". We have investigated the effect of interaction between *CYP2C9* and *VKORC1* (Chapter 6), the effect of polymorphisms in the gene that encodes for GATA-4, which is the transcription factor of CYP2C9 (Chapter 7), the effect of polymorphisms in *CYP3A4* and *CYP4F2* (Chapter 8), and the effect of statin use on the maintenance dose (Chapter 9). Although we

found some significant correlations between the investigated factors and coumarin maintenance dose, all results were of minor clinical impact. We did not find any interactions between the *CYP2C9* and *VKORC1* genotypes affecting the maintenance dose, time to severe overanticoagulation and time to achieve stability for phenprocoumon and acenocoumarol[29]. Mwinyi et al.[30] found that GATA-4 is a transcription factor of CYP2C9, the main metabolizing enzyme of coumarins. Therefore, it was hypothesized that polymorphisms in the gene encoding for this transcription factor might indirectly influence the coumarin dose requirements by influencing the amount of CYP2C9. We were the first research group investigating the effect of polymorphisms in the CYP2C9 transcription factor *GATA-4*. We found significant effects of polymorphisms in *GATA-4* on the acenocoumarol maintenance dose. The SNP rs3735814 explained an additional 1.1% of the acenocoumarol dose requirements in patients with the *CYP2C9* wild type genotype next to *VKORC1* genotype, age, height, weight, sex, and amiodarone use. For patients carrying one or more *CYP2C9* variant alleles, the rs3735814 SNP only explains an additional 0.5% of the acenocoumarol dose requirements, which was not significant. The additional dose variation explained was small and could not be replicated in a second independent cohort of patients using acenocoumarol. Polymorphisms in *GATA-4* therefore are not relevant for clinical implementation[31]. We also investigated the effect of 3 SNPs in *CYP3A4* (*CYP3A4*1B*, *CYP3A4*22*) and *CYP4F2* (*CYP4F2 V433M*) on the phenprocoumon and acenocoumarol maintenance dose. The phenprocoumon maintenance dose increased with 0.13 mg/day for patients carrying 1 *CYP4F2* variant allele and with 0.24 mg/day for patients carrying 2 variant alleles if compared to wild type patients (maintenance dose: 2.17 mg/day) (p=0.003). The phenprocoumon maintenance decreased with 0.18 mg/day for patients carrying 1 *CYP3A4*22* variant allele if compared to wild type patients (maintenance dose: 2.25mg/day). The *CYP3A4*1B* allele did not affect the phenprocoumon maintenance dose significantly. However, all significant interactions were not clinically relevant (additional square root dose variation explained was 0.8%). No effects were found for acenocoumarol (Chapter 8). The reason an effect was found for phenprocoumon and not for acenocoumarol might be that acenocoumarol is mainly metabolized by CYP2C9, while phenprocoumon is metabolized by several cytochrome P450 enzymes[32, 33]. We investigated the effect of statin use on the anticoagulant therapy and found that coadministration of statins with acenocoumarol leads to decreased acenocoumarol maintenance dosages, in particular simvastatin (-0.29 mg/day), pravastatin (-0.38 mg/day) and rosuvastatin (-0.69 mg/day), if compared to no statin use (maintenance dose: 2.60 mg/day). The phenprocoumon maintenance dose was not affected significantly. Physicians should be aware that concurrent use of statins with coumarins can increase the anticoagulation effect, especially in combination with acenocoumarol[34].

With our studies we unraveled a small part of the coumarin dose variability, but a large part is still unexplained. In addition, some factors are known or thought to influence the dose requirements, but are difficult to assess (e.g. vitamin K intake, stress), are unfeasible to include (e.g. fever), or only explain a small part of the dose variation (e.g. comedication use, SNPs in other genes than *VKORC1* and *CYP2C9*).

Strengths and limitations

The strength of our studies is the sample size of the Pre-EU-PACT cohort we used in most of the studies included in this thesis. We collected clinical and genetic information of 624 patients using phenprocoumon and 471 patients using acenocoumarol. With our smallest cohort, we were able to show an acenocoumarol dose difference of 0.035 mg/day with a power of 80% and a significance level of 0.05.

We collected the data in the Pre-EU-PACT cohort retrospectively. The advantage was that information of over 1000 patients could be collected in a short period of time. On the other hand, our approach might have introduced a selection bias since we included current phenprocoumon and acenocoumarol users. Therefore, long-term users are more likely to be selected. However, we did not see any differences in distribution of allele frequencies, amiodarone use, sex and age if compared with other studies[10, 25, 35].

For the validation of our results, we used cohorts of Dutch patients[26, 27, 36-38]. Therefore, it is uncertain whether our results are also applicable in other populations. As mentioned before, clinical and genetic data were comparably distributed in our Dutch cohorts as compared to other Caucasian populations. We therefore expect that the results will be valid in other Caucasian populations as well.

Implementation in clinical practice

Providing evidence

It is the current standard that randomized controlled trials (RCTs) determine the efficacy of therapeutic interventions before being implemented in clinical practice[39]. However, it will not be feasible to conduct an RCT for each newly discovered gene-drug interaction. There are several reasons for this. The first reason is that it is not always ethical to perform a clinical trial, for example in a situation in which observational studies have already shown that patients will be at high risk for an adverse event if they have a certain genotype[40]. Secondly, costs and resource use could be prohibitive. Performing clinical trials is very costly and it is not always clear

who should provide the money to conduct these trials. In most cases, pharmaceutical industry is not interested in financing the trials because the results of the trials may shrink their market by excluding part of the population from the use of their drug. Furthermore, a lot of this research is performed for drugs that have been on the market for some time, and might therefore no longer be under patent. Thirdly, clinical trials are time-consuming. For gene-drug interactions where observational evidence is convincing, it is not ethical to waste money and time. However, replication of the results in observational pharmacogenetic studies is often not obtained. Therefore, strict guidelines would help to define which evidence is necessary to implement the investigated pharmacogenetic interaction into clinical practice. Factors to consider are:

- Have the results been replicated in different studies?
- Are the results valid for various countries and ethnicities?
- Is the estimated improvement large enough?
- Is the estimated improvement cost-effective?
- Is it feasible to implement it in clinical practice? For example:
  - Are all facilities (e.g. genotyping instrument, guidelines) available?
  - Are the genotyping results available in time?
  - Are the parties involved trained to perform the implementation?

In addition, the Clinical Pharmacogenetics Implementation Consortium (CPIP) of the National Institutes of Health Pharmacogenomics Research Network develops peer-reviewed gene-drug guidelines to help clinicians how available genetic information of a patient can be used to optimize drug therapy[41-46]. The guidelines will include dosing recommendations, the quality of the evidence, and strength of evidence[47]. The CPIP will rate the quality of the evidence (e.g. study design, and number, power and consistency of the studies) with a three-tier scheme (level 1; good, level 2; sufficient, and level 3; insufficient). The strength of the recommendation is also evaluated using a three-tier rating scheme (A; strong, B; moderate, and C: optional). The cost-effectiveness analysis exceed the scope of the guidelines. The factors we thought to be considered are in general comparable to the factors the CPIP considers writing pharmacogenetics guidelines. In the Netherlands, a comparable consortium is operating; the Working Group Pharmacogenetics of the Royal Dutch Association for the Advancement of Pharmacy develops pharmacogenetic-based therapeutic (dose) recommendations[48, 49].

If RCTs are required to investigate the efficacy of pharmacogenetics interventions, optimizing the trial design might influence the efficiency of the trial[50, 51]. For example, an adaptive trial design could be beneficial. Adaptive trials enable the researcher to

implement prior information (from observational research, but also information obtained during the earlier phase of the trial itself) to optimize (the remainder of) the trial design. There are several possibilities for trial design adjustment. For example, sample size estimations might be revised based on interim analyses. The adaptive trial design is expected to have most success if certain subpopulations require adjusted treatment which is likely in the case of pharmacogenetic trials[50].

Parties involved in the implementation

When sufficient evidence is available for a clinical relevant gene-drug interaction, it should be implemented in clinical practice if it turns out to be cost-effective. There are multiple parties involved in the implementation of pharmacogenetic based therapies in everyday clinical situations. In this paragraph, we will discuss all different parties involved and their rationales.

Patients

Successful implementation of pharmacogenetic testing in everyday practice heavily depends on patient attitudes. Without the cooperation of patients, development of new pharmacogenetic strategies or guidelines is futile. Fortunately, research has shown that patients are willing to provide samples for genotyping. Van Wieren-De Wijer e*t al*. examined the reasons for non-participation in a pharmacogenetic case-control study[52]. They approached 1871 myocardial infarction cases and 14,102 controls of which 794 and 4997 responded, respectively. Approximately 40 to 46% of the patients did not return the questionnaire for private reasons or did not feel like returning the questionnaire. Only 1.1% of the non-participating patients were unwilling to provide a DNA sample[52]. This is in line with our experience of patient inclusion in the EU-PACT trial. We found that patients were very willing to participate and, in most cases, were able and willing to visit the anticoagulation clinic the first or second day of the coumarin treatment, the days they often were most ill. In addition, if patients are asked to be genotyped in situations where the clinical benefit has been proven instead of in the setting of a clinical trial, it is to be expected that more patients will agree to be genotyped.

Health care professionals

The attitude of health care providers towards pharmacogenetic guided therapies is important in making decisions about the treatment a patient will receive. Although the FDA updated the warfarin label already in 2007[53, 54], genotyping preceding anticoagulant therapy with coumarin derivatives is not routinely performed. Currently, health care professionals' attitudes are reserved towards pharmacogenetic dosing and pharmacogenetic-guided prescribing of drugs. Not many therapies need

pharmacogenetic testing at the moment, so health care professionals need to get familiar with the idea of genetic testing, like they are used to perform liver and kidney function tests. Different approaches are thought to help familiarize health care professionals with pharmacogenetic testing; clinical trials are needed to convince the health care professional and make genetic testing as normal as liver and kidney function tests. Moreover, recommendations in guidelines and drug labels of pharmacogenetic testing are required to improve treatment quality, such as the FDA did for warfarin. Furthermore, education of the health care professional on how to perform and use the pharmacogenetic tests is desired. Genotyping the patients with easy to use point-of-care tests could be performed by for example the general practitioner, nurse in the hospital or pharmacists. The results of, for example, CYP-enzymes genotypes, could be used for decision making in multiple therapies. Therefore, dissemination of the genotyping results (e.g. by means of electronic dossiers) is important so all health care professionals can individualize the patient's therapy when necessary and also avoid repeating genotyping the patient at different sites. Finally, to enhance the implementation of pharmacogenetic testing, the Royal Dutch Association for the Advancement of Pharmacy developed pharmacogenetic-based therapeutic (dose) recommendations[48, 49], presently already for 53 drugs.

Regulatory authorities

Regulatory authorities can also play an important role in the implementation of pharmacogenetic guided therapies in daily practice by developing guidelines. They can also adjust the label information of the medication.

The Committee for Human Medicinal Products (CHMP) facilitated an informal process of sharing scientific and technical information on pharmacogenetic data between applicants and regulators by releasing a concept paper on "Briefing Meetings on Pharmacogenetics"[55]. This guideline provides guidance for starting the discussion regarding the implementation of pharmacogenetic testing with the Pharmacogenetics Working Party and provides considerations on the submission of pharmacogenetic data in informal regulatory submissions. Briefing meetings take place when new pharmacogenetic information becomes available during the development of a new medicinal product or when a new indication is explored based on recent developments in pharmacogenetics. The Food and Drug Administration (FDA) developed a guideline called "Guidance for Industry, Pharmacogenomic Data Submissions". This guideline facilitates the scientific pharmacogenetics process and the use of pharmacogenetic data in drug development[56]. The FDA and European Medicinal Agency (EMA) have joint Voluntary Genomic Data Submissions (VGDSs). This is not part of the regulatory decision-making process, but gains an understand-

ing of genomic data and provides options for sponsors to have joint FDA-EMA briefing meetings[57].

Health insurance companies
Implementation of pharmacogenetic guided approaches to plan therapy will depend on whether the costs are reimbursed by health insurance companies. If the patient needs to pay for the genotyping kit, it is less likely that pharmacogenetic testing will be implemented in clinical practice than when health insurance companies will pay for it. However, these companies will likely only pay for genetic tests if their use leads to more cost-effective care. Health insurers would be very interested in genotyping if it improved treatment effectiveness but also reduced total health care costs, including the cost of genotyping. There are different ways in which genotyping results could lead to lower health care costs, for example fewer visits to the general practitioner or hospital for therapy adjustments (i.e. improved patient response or efficacy), better prophylaxis resulting in lower costs, and fewer side effects, especially serious side effects resulting in expensive hospital admissions.

In some cases, health insurers may reimburse genotyping even if it is believed to increase overall costs. For example, if the genotyping approach is more costly and more effective compared to the nongenotyping approach, the health insurer could consider the greater effectiveness worth the extra cost. All in all, this means that pharmaco-economic evaluations are of importance in pharmacogenetic studies. See also the paragraph on cost-effectiveness analysis.

Researchers
Sound scientific research is needed to develop new strategies of pharmacogenetic guided therapies. Both academia and pharmaceutical industry are involved in this research. Of course different study designs are possible. First, new pharmacogenetic interactions in existing drugs can be investigated. This is especially useful if it is clear that only part of the patients react to the drug, or if part of the patients suffer from a (serious) adverse drug reaction. Depending on their hypothesis, researchers could look for common SNPs that have a small effect, but since the SNPs are common, many patients might benefit. On the other hand, they could investigate rare SNPs that might cause major effects, in which case there could be a larger benefit for relatively few patients. However, this last area of research would require large sample sizes to have enough power to investigate the effect of a rare SNP. Second, studies to develop better and faster genotyping methods will be required if pharmacogenetic testing is to be used regularly in clinical practice. An example of a user-friendly and quick point-of-care genotyping test is the Optisense's Genie 1 with HyBeacon® assays

(see also Pharmacogenetic developments, subhead Point-of-care testing later in this Discussion)[58]. Third, the industry could develop new drug therapies for a genetic subpopulation. For example, a new drug that does not have the desired effect in the whole population might benefit patients with a certain genotype. Although only for this subpopulation, this new medication could then still enter the market. Finally, at this moment clinical trials are needed to convince health care professionals to implement pharmacogenetic testing in daily practice. However in the future, observational studies might provide enough evidence to be used for implementation. But because non-replication is very common in pharmacogenetic research, it is important that the results are replicated in various external data sets before being implemented in clinical practice. After implementation, it remains important to validate the outcomes and adjust the pharmacogenetic based guidelines, if necessary.

Facilities

Several facilities should be in place before pharmacogenetic testing can be implemented in clinical practice.

Availability of genotyping results

Genotyping results should be available quickly, especially for drugs that need to be prescribed immediately such as coumarins. If results are available before the therapy starts, they are of greater value than when they become available after treatment start. However, in the current clinical situation, health care professionals need to collect blood samples from a number of patients to be able to genotype a batch of samples. Therefore, it can sometimes take a few weeks before the genotype is known. Currently, new techniques are being developed (see also Pharmacogenetic developments, subhead Point-of-care testing later in this Discussion), and will continue to be developed in the coming years, to make genotyping results more rapidly available[58]. The need to collect samples from many patients will diminish, since one assay can be ran using a point-of-care test for a single patient. By increasing the number of assays needed, the availability of point-of-care test will increase[59] and the price per assay will probably decrease.

Authority guidelines

The authorities can assist in implementing pharmacogenetic testing in clinical practice by developing guidelines. In 2007, the FDA updated the warfarin label[53, 54] and advised pharmacogenetic testing before the coumarin therapy starts. However, at that time no guidelines were provided as to how the dosages should be changed based on the genetic profile of the patient. This illustrates that guidelines should

contain information on how to adjust drug therapy based on genotype. It also underlines the importance that different parties work closely together.

Cost-effectiveness analysis of pharmacogenetic testing

Decision making about the use of genotyping in clinical practice also depends on its cost-effectiveness. This means that even if authorities were to recommend genotyping patients prior to cardiovascular therapy based on proof of effectiveness, the recommendation might not easily be implemented without the support of other stakeholders. One important stakeholder is the payer, such as a health insurance company.

A cost-effectiveness analysis (CEA) compares the total costs and effectiveness of two or more treatment strategies. All sorts of costs must be considered here, including not just the cost of genotyping, but also the cost of monitoring and the cost of cardiovascular events that occur later in time. While costs are all expressed in the same way (money!), effectiveness can be defined in different ways. The definition of effectiveness determines how cost-effectiveness is expressed. For example, the difference in effectiveness between two treatments can be expressed as the absolute reduction in risk of an event. The cost-effectiveness of one treatment versus another will then be expressed as the extra cost to avoid one adverse event (calculated by dividing the difference in costs by the reduction in risk). However, since this expression of cost-effectiveness is very disease-specific, it is difficult, if not impossible, to compare the cost-effectiveness of different treatments for different diseases with each other and this comparability is valuable when making budget allocation decisions. For this reason, some authorities or health insurance companies require a cost-utility analysis (CUA). In a CUA, the health gains acquired by a new treatment are expressed in Quality Adjusted Life Years (QALYs), which can be compared more easily with other treatments, also in other diseases, than the cost per adverse event avoided.

Several economic evaluations (such as CEAs and CUAs) have been performed for coumarin derivatives. The problem with these analyses is that no robust data on the effectiveness of genotyping are available yet[60]; the large RCTs that can provide this data are still ongoing[21, 61]. A more reliable estimate of the cost-effectiveness or cost-utility of genotype-guided coumarin dosing can be calculated after the results of the large RCTs become available. The current lack of evidence results in a wide variability in cost-effectiveness ratios among the studies that have been done, ranging from US$60,750 to US$347,000 per QALY gained[60, 62, 63]. The costs of genotyping are also not clear yet. In literature, the estimated cost of genotyping for *CYP2C9* ranges from US$67 to US$350 and the estimated cost of genotyping both *CYP2C9* and *VKORC1*

ranges from US$175 to US$575[60, 62]. Recently, a point-of-care test for genotyping *CYP2C9* and *VKORC1* has been developed. With this test, the patient's genotype can be determined in the physician's office within 2 hours and this is estimated to cost less than US$50 per patient for both *CYP2C9* and *VKORC1*[58]. The costs of genotyping are expected to decrease even further, with increased usage. This will also increase the cost-effectiveness of pharmacogenetics.

Decisions about whether or not to implement pharmacogenetic testing in clinical practice will differ among different countries. This difference can be caused by several factors. Firstly, the amount of money society is willing to pay varies among different countries. For example, this 'willingness to pay' is approximately US$50,000 to US$100,000 per QALY gained in the US[64] or £20,000–30,000 (approximately $33,000-50,000) per QALY gained in the UK[65]. Secondly, the costs, not only of genotyping but also of the consequences like hemorrhage events, are not identical in all countries[66]. Next to this, the effectiveness of genotyping can also be higher in one country than in another because of the differences in standards of care. This is for example possibly the case with coumarin derivatives. In some countries the standard care is already of very high quality, with specialized anticoagulation clinics to monitor the effect of the drug, while in other countries this is not the case and there is therefore more room for improvement. Therefore it will also be necessary to carry out country-specific analyses in the future.

As mentioned before, the use of pharmacogenetics in treatment with a certain drug can only be recommended if information on effectiveness and costs of genotyping is available, although it is not clear what level of evidence is needed for a valid decision. Obviously, it is impossible to obtain perfect evidence. Therefore, value of information (VOI) analyses could be performed to establish the cost–effectiveness of further research on the efficiency of the strategy. If the costs of performing this research are higher than the benefits of the additional information, then it would not be worthwhile to conduct this research[67]. The parameters that have the largest influence on the uncertainty regarding the cost–effectiveness of genotyping should be the main focus of future studies in this area. The costs of conducting these studies should also be considered.

Pharmacogenetic developments

Common relevant SNPs will affect the therapy of more patients than rare SNPs will do. However, rare SNPs with large effects might as well be of importance, but it

might be a challenge to find large numbers of cases that are required to obtain enough power in pharmacogenetic studies[68]. A trend is observed that larger studies are being performed and meta-analyses are carried out to investigate these less frequent genetic profiles. Several techniques are further developed and might lead to new insights in the pharmacogenetic research field. We will discuss them in the following paragraphs.

Candidate gene studies

This type of study investigates the association between drug response and previously identified candidate genes. These candidate genes might play a relevant role in the pharmacokinetics or pharmacodynamics of the drug and might therefore be, for example, the metabolizing enzyme or the target protein. An example is the use of candidate gene approaches for the understanding of the overall drug response to coumarins[68]. In 1992, Rettie *et al*. indicated *CYP2C9* as main metabolizing enzyme of warfarin[69]. A few years later, Furuya *et al*. first reported that SNPs in this gene affect the stable coumarin maintenance dose[70]. A decade later, VKORC1 was identified as the target enzyme of the coumarins[71, 72] and studies confirming the association between *VKORC1* genotypes and stable coumarin maintenance dose followed. Another example is the role of the *CYP2C19* genotype on the clopidogrel[73] therapy response and how the treatment with tamoxifen is influenced by the *CYP2D6* genotype[74]. An advantage of candidate gene studies is that the biological mechanism is clear and smaller patient numbers might be needed to investigate an effect. A disadvantage is that only one association is investigated while more interactions might be relevant and therefore additional genetic effects might be missed.

Genome wide association studies

Since 2007, genome wide association (GWA) studies have become more frequently applied in the pharmacogenetics field. This resulted in novel identified associations between drug response and variations in DNA, but also confirmed already known associations[68]. For example, statin induced muscle symptoms was first found significantly associated with *SLCO1B1* in a GWA study[75] and for clopidogrel, the influence of *CYP2C19* on the effectiveness of the drug was confirmed[76]. In a GWA study on acenocoumarol maintenance dose, an additional effect was found for polymorphisms in *CYP4F2* and *CYP2C18*[77]. These GWA studies led to more knowledge about several drug-gene interactions, but the causality of the relationship is not always clear in these studies. A large advantage of performing a GWA study is that a lot of information on many genes is obtained, but one should keep in mind that this type of analyses need large patient numbers to find statistically significant associations because of the correction for multiple testing.

Sequencing

DNA sequencing is the determination of the nucleotide bases in DNA. In contrast to GWA studies, where tag SNPs are used to cover as much of the variation within the gene as possible, this technique will determine the exact order of nucleotides in DNA. Instead of tag SNPs that are usually markers for the causal SNP - and thereby introduce noise because they are not always in complete linkage disequilibrium - the causal SNPs can be identified. Therefore, this technique might provide new insights in associations between drug response and pharmacogenetic parameters that are not observed when performing a candidate-gene study or a GWA study. It is possible to sequence a whole genome or whole exome. In addition, there is an option 'targeted sequencing' which means that a candidate gene is sequenced. This technique is relatively new and gaining interest in the last few years, but the same issues (i.e. causality of the relationship is not always clear and large patient numbers are needed) as with the GWA studies occur with sequencing. This warrants that the functionality of the SNP should be studied[78].

Point-of-care testing

Point-of-care tests can be used as mobile genotyping instruments in different settings, including the pharmacy, anticoagulation clinic and physician's office. It avoids the need to collect samples of multiple patients and the genotyping results are available within 2 hours. This technique might be used to genotype the patient before the start of the therapy. However, the applicability of a point-of-care test may be different from centralized laboratory testing because of different sensitivity and specificity parameters. It is not attractive to use such a test in research where large patient groups are needed to find a pharmacogenetic interaction, since that would be very labor intensive.

We use a point-of-care test in the EU-PACT trial; Optisense's Genie 1 with HyBeacon® assays[58]. Although the assays are currently not always providing sufficient results in the first run and therefore sometimes need repetition, the genotypes obtained until now have been always in agreement with results obtained with the Taqman, a validated genotyping method. The robustness of the assays needs improvement before they can be used on larger scale. Genotyping a patient with this point-of-care test is very easy, it does not require a laboratory environment and no intensively trained personnel is required to use the system.

Developments in oral anticoagulant therapy

New oral anticoagulants (NOACs) are entering the market. In contrast to coumarins, which have an indirect mechanism of action by inhibiting vitamin K reduction and therefore reducing the synthesis of active clotting factors II (protrombin), VII, IX, and X[5], NOACs work directly on active clotting factors[79-83]. Dabigatran is a direct thrombin inhibitor[79], the other two NOACs that already entered the market are rivaroxaban and apixaban, which are direct factor Xa inhibitors[80-83]. In the Netherlands, dabigatran, rivaroxaban and apixaban are included in the Drug Reimbursement System (in Dutch: Geneesmiddelenvergoedingssysteem, GVS) for the prevention of thromboembolic events after hip- and knee replacement surgery[84]. In addition, dabigatran and rivaroxaban were registered in Europe for prevention of stroke and systemic embolism in patients with nonvalvular atrial fibrillation last year. Rivaroxaban is also registered for the treatment of deep vein thrombosis (DVT) and prevention of recurrent DVT and pulmonary embolism after acute DVT. Manufactures of both dabigatran and rivaroxaban requested for reimbursement for these indications as well; the decision is still pending.

These new oral anticoagulation drugs have some advantages but also several disadvantages when compared to the coumarins. We will first summarize the main disadvantages of the coumarins over NOACs and then summarize the most important advantages of coumarins.

Disadvantages coumarins over NOACs

In general, NOACs have been shown to have comparable or increased efficacy, fewer intracranial hemorrhages, but no difference in mortality (for apixaban lower mortality rates were found) if compared to coumarins when tested in a relatively healthy group of patients with atrial fibrillation[79, 80, 82]. Patients using 110 mg dabigatran twice daily, have similar rates of thromboembolic events to that of warfarin (relative risk: 0.91; p=0.34) while they suffered from lower major hemorrhages rates (relative risk: 0.80; p=0.003). When receiving 150 mg dabigatran twice daily, patients suffered less often from thromboembolic events (relative risk: 0.66; $p \leq 0.001$) and had an equivalent number of hemorrhages if compared to warfarin use (relative risk: 0.93;p=0.31). Administration of apixaban resulted in lower rates of thromboembolic events if compared to warfarin (hazard ratio: 0.79; p=0.01) as well as lower rates of hemorrhages (hazard ratio: 0.69; $p \leq 0.001$). Rivaroxaban was noninferior to warfarin regarding thromboembolic events (hazard ratio: 0.88; $p<0.001$ for noninferiority and p=0.12 for supriority) and showed no difference in risk of hemorrhage (hazard ratio: 1.03; p=0.44). However, one should keep in mind that the anticoagulant therapy

in the Netherlands is of high quality and future research should provide insight whether these improved safety results are also applicable for the Dutch patients.

Anticoagulant therapy with coumarins requires frequent monitoring of the International Normalized Ratio (INR) to find the optimal dose for each patient[85, 86]. This is because of the intra- and inter-patient variability in dose response dosages. Coumarin dosages may differ up to 10-fold between patients, but also vary over time in one individual. NOACs on the other hand can be prescribed to the patient using a fixed dose, i.e. for all patients the same dose and no need for titration[79, 80, 82]. This means that it is not required for the patient to have frequent monitoring appointments at the anticoagulation clinic, general practitioner's office or hospital for blood withdrawal to determine the INR. Another advantage of the NOACs is that they do not have many genetic, drug and food interactions as in the case with coumarins[87, 88].

Advantages coumarins over NOACs

The NOACs recently entered the market and therefore long-term adverse effects are still uncertain. Coumarins are already on the market for decades and therefore have less uncertainties. Caution is advised for unknown -long term- adverse effect of NOACs.

As discussed above, the advantage of NOACs over coumarins is that no longer frequent monitoring is required, but this might turn out to be an advantage for coumarins over NOACs. Most patients, especially with the indication of atrial fibrillation, do not feel they need an anticoagulant. Better compliance and adherence is expected for coumarin use. For example, in a study by Gomes *et al*., 8.9% of the patients did not fill a second warfarin prescription, 31.8% discontinued warfarin within 1 year, 43.2% in 2 years and 61.3% in 5 years[89]. With regular monitoring of the INR, patients are expected to have a better adherence to their oral anticoagulant if compared to the situation in which they are not monitored[90]. Currently, the anticoagulant effect of NOACs cannot be measured due to a lack of validated tests[87].

All anticoagulation drugs balance between thrombosis and hemorrhages. Besides these adverse effects, NOACs are associated with abnormal liver function tests[91] and an increased risk of myocardial infarction[79, 87]. In the studies, NOACs were tested in relatively healthy patients[79, 80, 82]. Usage of NOACs in clinical practice is required to evaluate the usage in relatively less healthy patients as well. For example, patients with lowered renal function were excluded from the NOAC trials (creatinine clearance lower than 25 or 30 ml/min)[79, 80, 82]. NOACs are contraindicated for patients with

renal dysfunction[92]. For these patients, coumarins probably remain the anticoagulant of first choice.

It is proven that anticoagulation clinic care increases the time spent in target INR range if compared with routine medical care[93]. In the Netherlands, an even higher percentage time in target INR range is found[5, 94]. Despite frequent monitoring – costs for blood withdrawal, INR measurements, and dosing by a physician- the coumarin therapy is cheaper than treatment of patients with a NOAC.

Finally, an antidotum exists for coumarins, but not (yet) for NOACs. In case a patient has a hemorrhage or needs acute surgery, it is important to antagonize the anticoagulant therapy. Up to date, no standardized antidotum is available for the NOACs[84]. One study showed reversal of rivaroxaban, but not dabigatran, on the prothrombin time after administration of prothrombin complex concentrate. However, one should keep in mind that this study was conducted in healthy volunteers and that the researchers looked at a proxy of the clinical outcome hemorrhage[95].

Perspective of oral anticoagulant therapy

NOACs are expected to be prescribed more often in the coming years. However, the part of the anticoagulation market that they will gain depends on a lot of factors and is currently uncertain. Nevertheless, it seems obvious that coumarins will remain to be prescribed, and for these patients it remains important to optimize the anticoagulant therapy.

Future research

We will finish inclusion of patients in the EU-PACT trial at the beginning of 2013. After that, we will investigate our primary outcome measure: will pretreatment genotyping of *VKORC1* and *CYP2C9* increase the percentage time spent within the target INR range during the first 12 weeks of the anticoagulant therapy? In addition, we will also assess the cost-effectiveness and quality of life of pretreatment genotyping, compare the safety and efficacy in both study arms, judge the clinical utility of the point-of-care test, look at the number of coumarin dose adjustments, and identify the incidence of coumarin sensitivity and resistance.

In this thesis, we have shown that a large part of the phenprocoumon and acenocoumarol maintenance dose can be explained by genetic and clinical information of a patient. However, still a large part of the variation (up to 40%) is not yet explained.

The plan is to sequence all patients being sensitive and resistant to the phenprocoumon (≤1.5 mg/day or ≥6mg/day) and acenocoumarol (≤1.0 mg/day or ≥8mg/day) anticoagulant therapy. Also, we have investigated only the effect of 7 SNPs in *GATA-4* on the phenprocoumon and acenocoumarol maintenance dose, but it is possible that there is another SNP in *GATA-4* that has a larger effect on the coumarin maintenance dose. It might be interesting to sequence this whole gene and further investigate the effects on the coumarin maintenance dose.

The safety and efficacy of NOACs have been compared to warfarin use. However, in The Netherlands acenocoumarol and phenprocoumon are used instead of warfarin. It would be interesting to compare the efficacy and safety of NOACs with these coumarins as well. Especially since the quality of the anticoagulant therapy in the Netherlands is higher than most other countries and therefore it is less likely to find an improvement of efficacy and safety for NOACs if compared to standard clinical care in the Netherlands. In addition, it is hypothesized that pretreatment genotyping will improve the quality even further. It might be interesting to compare the safety, efficacy and cost-effectiveness of the NOACs to the pretreatment genotyping approach with phenprocoumon and acenocoumarol in the Netherlands.

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Summary

Coumarin derivatives, such as warfarin, phenprocoumon and acenocoumarol, are effective in the prevention and treatment of thromboembolic diseases. Examples of indications are atrial fibrillation and venous thromboembolism. In the Netherlands, over 376,000 (2% of the population) patients used a coumarin derivative in 2010. Although coumarins are on the market for decades, it is still challenging to find the optimal dosage for each patient since coumarins have a small therapeutic index. In trying to achieve the optimal anticoagulant effect, the patient constantly balances between a too low anticoagulant effect -which increases the risk of thromboembolic events- and a too high anticoagulant effect -which increases the risk of hemorrhages. In addition, there is wide inter- and intra-patient variability in coumarin dose requirements. A dosage that provides optimal anticoagulation status in the first patient might cause hemorrhages in a second patient and thromboembolic events in a third. At the start of the anticoagulant therapy in current clinical practice, the dose of the first therapyday wil at most be reduced with a couple of milligrams for elderly, but in general, the physician will prescribe a universal coumarin loading dose to all patients while it is known that large inter-individual differences exist. Currently, the dosage is individualized by frequent monitoring of the blood parameter International Normalized Ratio (INR), which is a measure for anticoagulation status, followed by dose adjustments if required. In this thesis, we described studies that aimed to develop pretreatment personalized dosing strategies for coumarins.

In Chapter 1 we provided a general introduction on the history of coumarins, how they became an effective human drug after being a deadly cow poison. Moreover, we describe that pharmacogenetics (the study of variations in DNA sequence as related to drug response) enhanced the shift of an empiric approach of finding the optimal dose at coumarin treatment start to an approach in which new predictive factors are included in determining the individual dose before start of the therapy: personalized medicines.

We provided an introduction to the pharmacogenetics of oral anticoagulant therapy with coumarins in Part I, Chapter 2. The target enzyme of the coumarins is vitamin K epoxide reductase complex subunit 1 (VKORC1). Single Nucleotide Polymorphisms (SNPs) in the gene encoding for this protein explain the variability in coumarin dose requirements for approximately 25 to 35%. Other SNPs that explains coumarin dose requirement variability are located in the gene encoding for Cytochrome P450 2C9 (CYP2C9), the main metabolizing enzyme of the coumarins. However, with 4.5-17.5% of the variability being explained by SNPs in this gene its contribution is much lower than that of *VKORC1*. In addition, coumarin dose requirements are also influenced

by patient characteristics and environmental factors, such as concurrent use of medication, comorbidities, age, weight, height, and dietary vitamin K intake.

In Part II, we discussed the development and usage of the phenprocoumon and acenocoumarol dose algorithms. In Chapter 3 we described the development and validation of nongenotype- and genotype-guided dose algorithms for phenprocoumon and acenocoumarol. We included information on age, height, weight, sex, and amiodarone use in the nongenotype-guided algorithms. The genotype-guided algorithms included information on the *VKORC1* genotype and *CYP2C9* genotype in addition to age, height, weight, sex, and amiodarone use. The genotype-guided dose algorithm explained 55.9% and 52.6% of the variance of the maintenance dose, for phenprocoumon and acenocoumarol, respectively. The nongenotype-guided dose algorithm explained 17.3% and 23.7% of the dose variability, for phenprocoumon and acenocoumarol, respectively. The algorithms were validated in independent data sets and performed equally well in these cohorts. However, information on height and weight were missing in these validation cohorts. We described the validation of the acenocoumarol dose algorithms in a third data set that included information on height and weight in Chapter 4. The algorithms performed just as accurately in this study as in the original study.

It is known that coumarin dosages are influenced by polymorphisms in *VKORC1* and *CYP2C9*, age, weight, height, sex, and amiodarone use, but it is uncertain what the impact is on time within target INR range and clinical outcomes such as hemorrhages if this information is used to personalize the coumarin dose before start of the oral anticoagulant therapy. Therefore the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial was developed, of which the study design is presented in Chapter 5. In the EU-PACT trial, patients diagnosed with atrial fibrillation or deep vein thrombosis were randomized to either the intervention arm and therefore receiving a dosage based on the genotype-guided dosing algorithm, or to the control arm and therefore received a dosage based on the nongenotype-guided dosing algorithm. The *VKORC1* and *CYP2C9* genotype of the patient is determined with a point-of-care test that provids results within 2 hours and can be used in a non-laboratory environment. This single-blind trial with a follow-up period of 3 months assessed the safety and clinical utility of genotype-guided dosing in daily practice for the three main coumarins (warfarin, phenprocoumon and acenocoumarol) used in Europe.

Effects of genetic variance and comedication on the anticoagulant therapy are described in Part III. In Chapter 6 we evaluated a possible gene-gene interaction between *CYP2C9* and *VKORC1*. We investigated 3 different outcomes, namely the

maintenance dose, time to severe overanticoagulation (INR>6.0), and time to achieve stability. No significant interactions were found for all outcome measures for both phenprocoumon and acenocoumarol.

In Chapter 7 we evaluated the effect of genetic variations in *GATA-4*, the gene encoding for a CYP2C9 transcription factor, on the phenprocoumon and acenocoumarol maintenance dose. SNPs in *CYP2C9* affected the coumarin maintenance dose. GATA-4 regulates the transcription of CYP2C9 and it was hypothesized that polymorphisms in *GATA-4* affect the transcription of CYP2C9 and therefore the amount CYP2C9. Varying concentrations of CYP2C9 caused by *GATA-4* SNPs might further explain inter-patient variability in coumarin dose requirements. We indeed found an association between *GATA-4* SNPs and the acenocoumarol maintenance dose, however effects were small and the results could not be replicated in an independent data set. No significant association was found for phenprocoumon. Genetic variation in the 7 investigated *GATA-4* SNPs therefore do not seem relevant for clinical implementation.

Other SNPs that were hypothesized to affect the coumarin maintenance dose were SNPs in *CYP4F2* (metabolism vitamin K) and *CYP3A4* (metabolism coumarins). We described the evaluation of the effect of *CYP3A4*1B*, *CYP3A4*22* and *CYP4F2 V433M* on the phenprocoumon and acenocoumarol maintenance dose in Chapter 8. *CYP3A4*1B* is the most common variant allele, but in our study it was not associated with the phenprocoumon or acenocoumarol maintenance dosages. *CYP3A4*22* had been recently identified as new functional SNP. This SNP has a marginally significant effect on the phenprocoumon maintenance dose. CYP4F2 plays a role in the metabolism of vitamin K. Patients carrying a variant allele have a reduced capacity to metabolize vitamin K if compared to non-carriers, resulting in increased vitamin K levels and thus higher coumarin dose requirements. For phenprocoumon, indeed a significantly increased maintenance dose was found for patients carrying *CYP4F2* variant alleles. The same trend was observed for acenocoumarol, but it was not significant. The clinical relevance of these 3 genotypes for personalizing the phenprocoumon and acenocoumarol dose is low.

In the Netherlands, over 10% of the population used a statin in 2010. Simultaneous use with coumarins will occur regularly. However, the effect of statin use on the phenprocoumon and acenocoumarol maintenance dose was not clear. Therefore we evaluated the effect of statin use on the phenprocoumon and acenocoumarol maintenance dose in Chapter 9. We found decreased acenocoumarol maintenance dose requirements when patients used concurrently either atorvastatin, simvastatin,

pravastatin or rosuvastatin. Therefore, we advise physicians to take into account the effect of statin use on the anticoagulant therapy with acenocoumarol. We did not find an effect on the phenprocoumon maintenance dose requirements.

In Chapter 10 we elaborated on the studies described in this thesis. The main findings were discussed and placed in a broader perspective. It was argued how to implement pharmacogenetics in a clinical setting and which parties are involved. Moreover, it was discussed how pharmacogenetics studies may provide evidence for implementation. The facilities required for implementation were considered and the cost-effectiveness and technical developments in pharmacogenetics were discussed. Finally, recent developments regarding the new oral anticoagulants (NOACs) were discussed and suggestions for future research were provided.

Samenvatting

Coumarinederivaten zoals warfarine, fenprocoumon en acenocoumarol zijn effectief voor de preventie en behandeling van trombo-embolische aandoeningen. Indicaties zijn bijvoorbeeld atriumfibrilleren en diep veneuze trombose. In 2010 waren er in Nederland 376.000 mensen die een coumarine gebruikten. Dat is ongeveer 2% van de Nederlandse bevolking. Hoewel coumarines al decennia op de markt zijn, is het nog steeds een uitdaging om de juiste dosering voor iedere patiënt te bepalen. Dat komt door het smalle therapeutische venster van de coumarines. Om het juiste antistollingseffect te bewerkstelligen balanceren patiënten tussen een te laag antistollingseffect (wat het risico op trombose verhoogt) en een te hoog antistollingseffect (wat het risico op bloedingen verhoogt). Daarnaast zijn er grote verschillen in coumarine dosisbehoefte tussen, maar ook binnen patiënten. Een dosering die het juiste antistollingsniveau geeft in de ene patiënt, zou bloedingen kunnen veroorzaken in een tweede patiënt en trombose in een derde. Bij start van de antistollingsbehandeling wordt in de huidige klinische praktijk bij de meeste patiënten dezelfde oplaaddosering voorgeschreven terwijl het bekend is dat er grote verschillen tussen patiënten zijn. De dosering wordt vervolgens geïndividualiseerd doordat patiënten regelmatig een International Normalized Ratio (INR, dat is een bloedwaarde die de mate van antistolling weergeeft) laten bepalen en aan de hand van deze bloedwaarde wordt de dosering aangepast. In dit proefschrift beschrijven we studies die als doel hadden om doseringsstrategieën te ontwikkelen die de individuele dosering voorspellen vóór de start van de behandeling met coumarines.

In Hoofdstuk 1 is een algemene introductie gegeven over de geschiedenis van de coumarines, hoe deze geneesmiddelgroep van dodelijk koeienvergif een effectief medicijn voor mensen werd. Tevens beschreven we hoe farmacogenetica (de studie van variatie in DNA gerelateerd aan geneesmiddelrespons) de verschuiving heeft teweeggebracht van een empirische benadering van doseren (trial- and- error) bij aanvang van de orale antistollingbehandeling naar een aanpak waarbij nieuwe voorspellende factoren bij aanvang van de behandeling al worden meegenomen: personalized medicine (gepersonaliseerde geneeskunde).

Het 1e deel, Hoofdstuk 2, is een introductie over de farmacogenetica van de orale antistollingsbehandeling met coumarines. Het aangrijpingsenzym van coumarines is vitamine K epoxide reductase complex 1 (VKORC1). Single Nucleotide Polymorphisms (SNPs) in het gen dat voor dit enzym codeert verklaren variabiliteit in de coumarine dosisbehoefte voor ongeveer 25 tot 35%. Andere SNPs die een rol spelen bij het verklaren van de coumarine dosisbehoefte zijn gepositioneerd in het gen dat codeert voor cytochroom P450 2C9 (CYP2C9), het voornaamste metaboliserend enzym van de coumarines. Het percentage van de variatie in dosisbehoefte dat SNPs

in dit gen verklaren is met 4,5 tot 17,5% veel lager dan de bijdrage van *VKORC1*. Naast genetische variatie in deze enzymen spelen karakteristieken van de patiënt en omgevingsfactoren zoals co-medicatie, comorbiditeiten, leeftijd, lengte, gewicht, geslacht en vitamine K inname ook een rol.

In het 2e deel worden de ontwikkeling en het gebruik van de fenprocoumon en acenocoumarol dosis algoritmes besproken. In Hoofdstuk 3 beschreven we de ontwikkeling en validatie van de niet-genotype- en genotype-geleide dosis algoritmes voor fenprocoumon en acenocoumarol. Informatie over leeftijd, lengte, gewicht, geslacht en amiodaron gebruik werden opgenomen in het niet-genotype-geleide dosis algoritme. Het genotype-geleide dosis algoritme bevatte naast de informatie die is opgenomen in het niet-genotype-geleide dosis algoritme ook informatie over het *VKORC1* en *CYP2C9* genotype van de patiënt. Het genotype-geleide dosis algoritme verklaarde 55,9 en 52,6% van de dosis variabiliteit voor respectievelijk fenprocoumon en acenocoumarol. Bij het niet-genotype-geleide dosis algoritme was dat 17,3% en 23,7%, voor respectievelijk fenprocoumon en acenocoumarol. De algoritmes werden vervolgens gevalideerd in onafhankelijk datasets waar ze vergelijkbaar goed in presteerden. In deze datasets ontbrak echter lengte en gewicht van de patiënten. Daarom werden de algoritmes nogmaals gevalideerd in een andere onafhankelijke dataset waarin wel informatie over lengte en gewicht beschikbaar is. Dit staat beschreven in Hoofdstuk 4. Ook hierin was de prestatie van de algoritmes net zo nauwkeurig als in de originele studie.

Het is dus bekend dat de coumarine dosering beïnvloed wordt door polymorfismes in *VKORC1* en *CYP2C9*, leeftijd, gewicht, lengte, geslacht en amiodaron gebruik. Het is echter niet bekend wat de invloed is op het percentage dat de INR van de patiënt zich binnen het INR streefgebied bevindt en op klinische uitkomsten zoals bloedingen als deze parameters worden meegenomen in het bepalen van de individuele coumarine dosis vóór de start van de antistollingsbehandeling. Daarom werd de European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial opgezet, waarvan de studieopzet is beschreven in Hoofdstuk 5. Patiënten gediagnostiseerd met atriumfibrilleren of diepe veneuze trombose werden in de EU-PACT trial gerandomiseerd in óf de interventie arm (deze patiënten werden gedoseerd op basis van het genotype-geleide algoritme) óf in de controle arm (deze patiënten werden gedoseerd op basis van het niet-genotype-geleide algoritme). Het *VKORC1* en *CYP2C9* genotype van de patiënt werd bepaald met behulp van een point-of-care test die de resultaten binnen 2 uur verschaft en die gebruikt kan worden buiten het laboratorium. Deze enkel blinde studie met een studieperiode van 3 maanden zal de veiligheid en klinische bruikbaarheid van genotype-geleid doseren in de dagelijkse

praktijk bepalen voor de 3 voornaamste coumarines die in Europa gebruikt worden; warfarine, fenprocoumon en acenocoumarol.

Effecten van genetische variatie en gebruik van andere medicatie naast de coumarine op de antistollingsbehandeling zijn beschreven in het 3e deel. In Hoofdstuk 6 evalueerden we een mogelijke gen-gen interactie tussen *CYP2C9* en *VKORC1*. We evalueerden 3 uitkomstmaten, namelijk de onderhoudsdosering, tijd tot ernstige overantistolling (INR>6.0), en tijd tot stabiliteit. We hebben geen significante interacties gevonden voor alle uitkomstmaten, voor zowel fenprocoumon als acenocoumarol.

In Hoofdstuk 7 is de evaluatie van genetische variatie in *GATA-4*, een CYP2C9 transcriptie factor, op de fenprocoumon en acenocoumarol onderhoudsdosering beschreven. SNPs in *CYP2C9* beïnvloeden de coumarine dosisbehoeften. GATA-4 zorgt voor de transcriptie van CYP2C9 en daarom werd verondersteld dat polymorfismes in *GATA-4* de transcriptie van CYP2C9 en daarmee dus ook de hoeveelheid CYP2C9 konden beïnvloeden. Variërende CYP2C9 concentraties veroorzaakt door SNPs in *GATA-4* zouden dus de inter-patiënt dosis variabiliteit verder kunnen verklaren. In onze studie vonden we een associatie tussen *GATA-4* SNPs en de acenocoumarol onderhoudsdosering, maar de effecten waren klein, de resultaten konden niet gerepliceerd worden in een onafhankelijke dataset en geen significante associatie werd gevonden bij patiënten die fenprocoumon gebruikten. Genetische variatie in de 7 onderzochte *GATA-4* SNPs lijkt daarom niet relevant voor implementatie in de klinische praktijk.

Andere SNPs waarvan gedacht werd dat ze invloed konden hebben op de coumarine onderhoudsdosering waren SNPs in *CYP4F2* en *CYP3A4*. In Hoofdstuk 8 beschreven we de evaluatie van effecten van *CYP3A4*1B*, *CYP3A4*22* en *CYP4F2 V433M*. Andere metaboliserende enzymen dan CYP2C9 spelen ook een rol bij het metabolisme van de coumarines. *CYP3A4*1B* is de meest voorkomende variant allel in CYP3A4. In onze studie bleek deze SNP echter niet geassocieerd met de coumarine onderhoudsdosering. Recentelijk is *CYP3A4*22* geïdentificeerd als functionele SNP. Deze SNP bleek in onze studie een marginaal significant effect te hebben op de fenprocoumon, maar niet op de acenocoumarol onderhoudsdosering. CYP4F2 speelt een rol in het metabolisme van vikamine K. Patiënten met een variant allel hebben een verlaagde capaciteit om vitamine K te metaboliseren in vergelijking met mensen die het wild-type allel hebben. Dit resulteert in verhoogde vitamine K concentraties en daarom ook hogere coumarine dosisbehoeften voor patiënten met variant allelen. Voor zowel fenprocoumon als voor acenocoumarol vonden we inderdaad verhoogde

coumarine dosisbehoeften bij patiënten met 1 of 2 variant allelen in *CYP4F2*, het effect was echter alleen significant voor fenprocoumon. Klinische relevantie van deze 3 genotypes om de coumarine dosering verder te individualiseren is laag.

In Nederland gebruikte in 2010 meer dan 10% van de populatie een statine. Gelijktijdig gebruik met coumarines komt regelmatig voor. Het effect van statine gebruik op de fenprocoumon en acenocoumarol dosering was echter onduidelijk. In Hoofdstuk 9 beschreven we daarom onze studie die als doel had het effect van statine gebruik op de fenprocoumon en acencoucoumarol dosering te onderzoeken. We vonden verlaagde acenocoumarol dosisbehoeften bij gelijktijdig gebruik van atorvastatine, simvastatine, pravastatine of rosuvastatine. We vonden geen effect op de fenprocoumon dosisbehoeften.

Hoofdstuk 10 is de algemene discussie van dit proefschrift. De resultaten van de studies worden in de discussie samengevat en in een breder perspectief geplaatst. Er wordt beschreven waaraan gedacht zou moeten worden bij het implementeren van farmacogenetica in de klinische praktijk en welke partijen daarbij betrokken zullen zijn. De faciliteiten die aanwezig moeten zijn voor de implementatie van farmacogenetica in de klinische praktijk worden geëvalueerd en de kosteneffectiviteit en technische ontwikkelingen op het gebied van farmacogenetica besproken. Tenslotte wordt er ingegaan op de recente ontwikkeling wat betreft de nieuwe orale antistollingsmiddelen (NOACs) en worden suggesties gegeven voor toekomstig onderzoek.

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List of publications

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van Schie RM, Wessels JA, Verhoef TI, Schalekamp T, le Cessie S, van der Meer FJ, Rosendaal FR, Visser LE, Teichert M, Hofman A, Buhre PN, de Boer A, Maitland-van der Zee AH. Evaluation of effects of genetic variations in GATA-4 on the phenprocoumon and acenocoumarol maintenance dose. *Pharmacogenomics* 2012 Dec; 13(16):1917-23

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Rianne van Schie was born on 14 April 1985 in Nieuwkoop, The Netherlands. In 2003, she graduated from secondary school "Alkwin Kollege" in Uithoorn. In the same year she started her study Biopharmaceutical Sciences with the track Science Based Business at the Leiden University where she obtained her Master of Science degree with distinction (cum laude) in 2008. In November 2008, she started the PhD research described in this thesis at the Division of Pharmaco-epidemiology and Clinical Pharmacology at Utrecht University. The topic of her research project was the pharmacogenetics of coumarin derivatives. An important part of the project was the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial, for which she included patients at the Anticoagulation Clinics Leiden, The Hague and Hoofddorp in The Netherlands. During her PhD-project, she received the Young Scientists Award at the "*Santorini Conference Biologie Prospective*" in Santorini, Greece (30 September - 2 October 2012).