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On the Prediction of Warfarin Dose

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Abstract
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Warfarin is one of the most widely used anticoagulants in the world. Treatment is complicated by a large inter-individual variation in the dose needed to reach adequate levels of anticoagulation i.e. INR 2.0 – 3.0. The objective of this thesis was to evaluate which factors, mainly genetic but also non-genetic, that affect the response to warfarin in terms of required maintenance dose, efficacy and safety with special focus on warfarin dose prediction.

Through candidate gene and genome-wide studies, we have shown that the genes CYP2C9 and VKORC1 are the major determinants of warfarin maintenance dose. By combining the SNPs CYP2C9 *2, CYP2C9 *3 and VKORC1 rs9923231 with the clinical factors age, height, weight, ethnicity, amiodarone and use of inducers (carbamazepine, phenytoin or rifampicin) into a prediction model (the IWPC model) we can explain 43 % to 51 % of the variation in warfarin maintenance dose. Patients requiring doses < 29 mg/week and doses ≥ 49 mg/week benefitted the most from pharmacogenetic dosing. Further, we have shown that the difference across ethnicities in percent variance explained by VKORC1 was largely accounted for by the allele frequency of rs9923231. Other novel genes affecting maintenance dose (NEDD4 and DDHD1), as well as the replicated CYP4F2 gene, have small effects on dose predictions and are not likely to be cost-effective, unless inexpensive genotyping is available.

Three types of prediction models for warfarin dosing exist: maintenance dose models, loading dose models and dose revision models. The combination of these three models is currently being used in the warfarin treatment arm of the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) study. Other clinical trials aiming to prove the clinical validity and utility of pharmacogenetic dosing are also underway.

The future of pharmacogenetic warfarin dosing relies on results from these ongoing studies, the availability of inexpensive genotyping and the cost-effectiveness of pharmacogenetic driven warfarin dosing compared with new oral anticoagulant drugs.

Keywords: Warfarin, pharmacogenetics, prediction models, Dosing algorithm, GWAS, VKORC1, CYP2C9

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To the patient

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Wadelius M*, Chen LY*, Lindh JD*, **Eriksson N***, Ghori MJ, Bumpstead S, Holm L, McGinnis R, Rane A, Deloukas P. (2009) The largest prospective warfarin-treated cohort supports genetic forecasting. *Blood*, 113(4):784-92

II Klein TE, Altman RB, **Eriksson N**, Gage BF, Kimmel SE, Lee MT, Limdi NA, Page D, Roden DM, Wagner MJ, Caldwell MD, Johnson JA. (2009) Estimation of the warfarin dose with clinical and pharmacogenetic data. *N Engl J Med.*, 360(8):753-64

III Limdi NA, Wadelius M, Cavallari L, **Eriksson N**, Crawford DC, Lee MT, Chen CH, Motsinger-Reif A, Sagreiya H, Liu N, Wu AH, Gage BF, Jorgensen A, Pirmohamed M, Shin JG, Suarez-Kurtz G, Kimmel SE, Johnson JA, Klein TE, Wagner MJ. (2010) Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups. *Blood*, 115(18):3827-34

IV **Eriksson N**, Wallentin L, Berglund L, Axelsson T, Connolly S, Eikelboom J, Ezekowitz M, Oldgren J, Pare G, Reilly P, Siegbahn A, Syvanen AC, Wadelius C, Yusuf S, Wadelius M. Genetic determinants of warfarin response, efficacy and safety in the RE-LY genomics substudy. Manuscript.

V **Eriksson N**, Wadelius M. Prediction of warfarin dose; why, when and how? (2012) *Pharmacogenomics*, 13, 429-40

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* These authors contributed equally to the work

List of Papers not included in this thesis

Papers not included in this thesis. These papers are referred to in the text by their capitalized letter.

A. Wadelius M, Chen LY, Downes K, Ghori J, Hunt S, **Eriksson N**, Wallerman O, Melhus H, Wadelius C, Bentley D, Deloukas P. (2005) Common VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics J.* 5(4):262-70.

B. Wadelius M, Chen LY, **Eriksson N**, Bumpstead S, Ghori J, Wadelius C, Bentley D, McGinnis R, Deloukas P. (2007) Association of warfarin dose with genes involved in its action and metabolism. *Hum Genet.* Mar;121(1):23-34.

C. Pare G, **Eriksson N**, Lehr T, Connolly S, Eikelboom J, Ezekowitz M, Axelsson T, Haertter S, Oldgren J, Reilly P, Siegbahn A, Ann-Syvanen A-C, Wadelius C, Wadelius M, Zimdahl-Gelling H, Yusuf S, Wallentin L. Genetic determinants of dabigatran plasma levels and their relation to clinical response. Manuscript.

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Abbreviations

A	Adenine
AF	Atrial fibrillation
AMI	Acute myocardial infarction
C	Cytosine
DNA	Deoxyribonucleic acid
F	Factor (coagulation factors)
HWE	Hardy-Weinberg equilibrium
G	Guanine
ICH	Intra cranial hemorrhage
INR	International normalized ratio
ISI	International sensitivity index
ISTH	International Society on Thrombosis and Haemostasis
IWDRC	International Warfarin Dose Refinement Collaboration
IWPC	International Warfarin Pharmacogenetics Consortium
LD	Linkage disequilibrium
MAF	Minor allele frequency
NCBI	National Center for Biotechnology Information
NNT	Number needed to treat
NNG	Number needed to genotype
NOAC	New oral anticoagulant
OOR	Out-of-range
PT	Prothrombin time
RNA	Ribonucleic acid
mRNA	Messenger RNA
SNP	Single nucleotide polymorphism
SS	Sums of squares
T	Thymine
TF	Tissue factor
TIA	Transient ischaemic attack
TTR	Time in therapeutic treatment range
VKA	Vitamin-K antagonists
vWF	Von Willebrand factor
WARG	Warfarin genetics (study)

Introduction

Oral anticoagulants have proven to be effective in lowering the risk of thrombo-embolic events in a wide range of disease conditions. Among these are prevention of systemic embolism in patients with heart valves or atrial fibrillation (AF), treatment of deep venous thrombosis or pulmonary embolism, prevention of venous thromboembolism after hip surgery and major gynecologic surgery, prevention of acute myocardial infarction (AMI) in patients with peripheral arterial disease and prevention of stroke, recurrent infarction, or death in patients with AMI.[1]

AF is the most common indication for oral anticoagulant treatment. AF is associated with an increased risk of stroke, thrombo-embolic events, heart failure and shortened survival.[2] Typical symptoms of AF are irregular or rapid heartbeat. Over 6 million Europeans suffered from AF in 2010 and the prevalence of AF increases with age, from <0.50 % at 40-50 years to 5-15 % at 80 years.[2] At present the prevalence is 1-2 %, but is expected to double in the next 50 years due to the aging population.[2] About one third of the patients have silent AF, i.e. they are without symptoms before detection by electrocardiography. In these patients AF may manifest itself initially as an ischaemic stroke or a transient ischaemic attack (TIA).[2] Hence, the true prevalence of AF is probably closer to 2 % of the population. The natural time course of AF starts with silent, short periods of AF, to progress to paroxysmal, persistent, long-standing persistent and permanent AF. Ischaemic strokes caused by AF are often fatal or disabling, thus stroke prevention in AF is an important field of research.[2]

Untreated, patients with AF have an average stroke rate of 4.4 % per patient year. However, the risk of stroke is strongly related to the patient's age and manifestations of cardiovascular disease. When sub-classifying patients into stroke risk groups according to the $CHADS_2$ classification system the crude stroke rates might range from 1.2 % to 8.0 % (Table 1).[3] The $CHADS_2$ classification system assigns one point for any of the following conditions; Congestive heart failure, Hypertension, Age at least 75 years or Diabetes mellitus and two points for having a prior Stroke or TIA.

Oral anticoagulants are commonly used to prevent strokes in patients with AF. For over 50 years the only available oral anticoagulants have been Vitamin-K antagonists (VKA). In AF, the risk of stroke is substantially reduced by VKA, such as warfarin. In a meta-analysis including 29 clinical trials on patients with non-valvular AF from 1966 to 2007, the relative risk reduction

of stroke with VKA compared to placebo was 64 % corresponding to an absolute annual risk reduction in all strokes of 2.7 %.[4] In recent clinical studies the incidence of stroke in patients with AF and at least one risk factor for stroke treated with warfarin was around 1.6 % per patient year (Table 3 on page 19).[5-7]

Table 1. *Estimated risk of stroke in the National Registry of Atrial Fibrillation, stratified by the $CHADS_2$ score.*

$CHADS_2$ score	Patients (n=1728)	Crude stroke rate per 100 patient-years	Adjusted stroke rate (%/year) with 95% confidence interval
0	120	1.2	1.9 (1.2-3.0)
1	463	2.8	2.8 (2.0-3.8)
2	523	3.6	4.0 (3.1-5.1)
3	337	6.4	5.9 (4.6-7.3)
4	220	8.0	8.5 (6.3-11.1)
5	65	7.7	12.5 (8.2-17.5)

Table adapted from Gage BF et al.[3]

Although there are positive effects in stroke reduction with oral anticoagulants, there is also a downside. Oral anticoagulants affect the clotting of blood, thereby prolonging the normal time it takes for blood to coagulate. Not surprisingly a common side effect of treatment with oral anticoagulants is risk of bleeding. The consensus today is that the positive effects on risk reduction of stroke outweighs the risk of major bleeding, hence treatment with oral anticoagulants is recommended for AF patients with at least one risk factor for stroke.[4]

This thesis is on the pharmacogenetics of the VKA warfarin. One of the challenges with warfarin treatment is the large inter-individual variation in dose needed to reach adequate levels of anticoagulation. The variation is more than ten-fold, ranging from less than 10 mg/week to more than 100 mg/week. The aim of this thesis is to evaluate which factors, both genetic and non-genetic, that affect the response to warfarin in terms of required maintenance dose, efficacy and safety with special focus on warfarin dose prediction.

Anticoagulation

The purpose of haemostasis is to maintain circulatory flow by keeping blood within a damaged blood vessel to prevent blood loss. It is a balance between procoagulant and anticoagulant mechanisms or pathways. Damage to the vasculature will initiate haemostasis, forming an impermeable platelet and

fibrin plug at the site of injury. The clot is later dissolved by a process called fibrinolysis.[8]

Although the process of haemostasis involves different stages, oral anticoagulants mostly act on coagulation factors involved in the cell surface based coagulation process (*Figure 1*). This process is a complex interaction between several coagulation factors resulting in circulating fibrinogen being transformed to fibrin, which is the key constituent of a blood clot. The coagulation process can be divided into overlapping stages; initiation, amplification and propagation.[8] The initiation phase starts with tissue factor (TF) binding to factor (F) VIIa, the TF/FVIIa complex then activates FX and FIX to FXa and FIXa. Subsequently, FXa generates trace amounts of thrombin (FIIa) from prothrombin (FII) that signal further platelet activation and aggregation in the amplification phase activating FV, FVIII (bound to von Willebrand factor, vVF) and FXI on the platelet surface. In the propagation phase the activated platelets are cofactors for the activation of the FVIIIa-FIXa complex (Xase) and the FVa-FXa complex (prothrombinase) resulting in a burst of thrombin involved in the process of converting fibrinogen to fibrin. Fibrin then forms a network of strings that, together with platelets, produces a clot at the wound site.

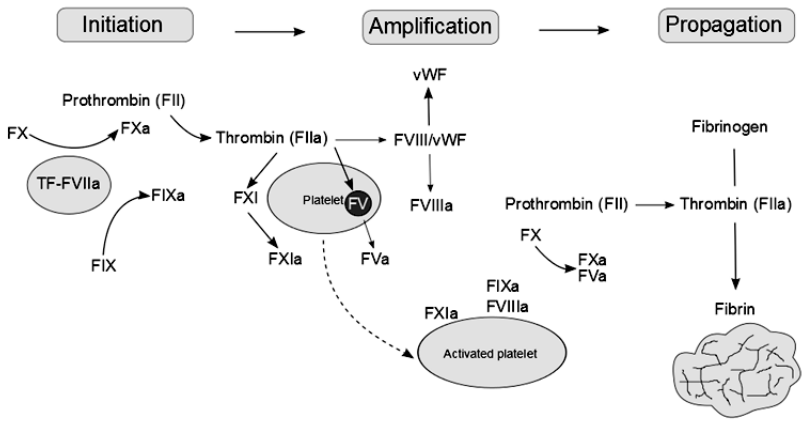

Figure 1. The coagulation process can be divided into three overlapping phases; initiation, amplification and propagation. Figure adapted from De Caterina, R et al.[8]

Oral anticoagulant drugs and their pharmacodynamics

Although VKA has been used clinically for nearly 60 years, it is only until the last two years that new alternatives to VKA have become clinically available. Today, the available oral anticoagulant drugs can be divided into three groups, VKA, factor Xa inhibitors and direct thrombin inhibitors.

VKA are warfarin, acenocoumarol and phenprocoumon. Warfarin is the most commonly used VKA with over 25 million prescriptions in the United States during 2010.[9] VKA targets the vitamin K cycle in the liver, thereby inhibiting the production of the coagulation factors II, VII, IX and X which are critically dependent on the levels of vitamin K.[10]

The oral factor Xa inhibitors, rivaroxaban and apixaban, are specific direct inhibitors of FXa-activity both in the fluid phase and in the prothrombinase complex.[8] One molecule of FXa catalyzes the conversion of more than 1000 molecules of thrombin. Through animal studies it has been suggested that inhibition of FXa causes less bleeding than direct thrombin inhibition.[11]

The direct thrombin inhibitor dabigatran is administered orally as dabigatran etexilate. This prodrug is rapidly transformed to the active entity dabigatran by serum esterase when entering the blood stream.[12] Dabigatran inhibits both free and clot-bound thrombin by binding to the active site of the molecule.[11]

The new anticoagulants can be used in fixed doses once or twice daily without need for laboratory monitoring of coagulation activity. The response to these drugs seems to be less dependent on the patient's genetic constitution and intake of food items and co-medications. All agents are at least partly eliminated renally why the doses need to be reduced in patients with renal impairment. In the pivotal randomized trials in patients with AF and at least one risk factor for stroke these new oral anticoagulants had as good or better protection against stroke, fewer intracranial bleedings and a trend to improved survival in comparison with warfarin dosed to maintain international normalized ratio (INR) at 2.0- 3.0.[5-7]

Although these new alternatives to VKA now are becoming available, currently warfarin remains as the most commonly used oral anticoagulant.[9]

Warfarin

Warfarin history

"Dere's no clot in that blook! BLUT, BLUT VERFLUCHTES BLUT" said Karl Links German student Mr. Shoeffel.[13] This was in 1933 and Shoeffel had just dipped his hands in a milk can full of blood. The blood came from a dead cow that a farmer had brought to the lab after it had died by something called the sweet clover disease. The sweet clover disease caused massive bleedings in cattle and was first spotted in the 1920's in the area around North Dakota. Later the cause of bleeding was traced to stacks of sweet clover hay that had gone bad, hence its name. In 1939 Karl Link and his research group managed to extract the anticoagulant dicumarol out of spoiled

sweet clover hay and in 1940 they created a synthetic product that was shown to be chemically identical. During 1941 – 1944 fifty reports on the clinical use of dicumarol appeared but it was not until 1954 that the more potent version, warfarin, appeared on the market. The sponsor of the project was the Wisconsin Alumni Research Foundation; the initial letters combined with "arin" from coumarin gave the substance its name. Warfarin was mainly developed for rodent control inferring skepticism among physicians and patients. It is said that one of the first patients to use warfarin was Dwight. D. Eisenhower, the president of the United States at that time.

Monitoring and quality control of warfarin treatment

The effect of warfarin is monitored by the INR value which is a standardized measure of the effects of VKA on clotting activity in the blood.

One problem with warfarin therapy is the large interindividual variation in the dose needed to reach therapeutic levels of anticoagulation; the variability in dose requirement is more than ten-fold, ranging from less than 10 to over 100 mg per week. This is further complicated by the established narrow therapeutic treatment interval, INR 2.0 to 3.0, which is needed to minimize both bleeding and thrombo-embolic events. Frequent INR monitoring and dose alteration is therefore necessary especially during the initiation of warfarin treatment and at temporary treatment interruptions.

Monitoring by INR was proposed by the World Health Organization (WHO) in 1982. Before this time the prothrombin time (PT) was used. PT is measured in seconds and the test is performed by adding calcium and thromboplastin to citrated plasma.[8] The PT test was developed by Quick in 1935 and it is sensitive to the presence and activity of FII, FV, FVII, FX and fibrinogen[1].[14] In the beginning it had some significant drawbacks; the main problem was lack of standardization which led to different results when comparing laboratories. The most alarming effect of the non-standardized PT method was that a patient could get different doses depending on where the PT was analyzed.[15] The problem was shown to be caused by the use of different thromboplastins which vary in responsiveness to reduction in the vitamin-K dependent coagulation factors.[8]

INR, sometimes referred to as PT-INR, is standardized by dividing the PT of a patient with the geometric mean of PT for at least 20 healthy subjects with the same test system and adjusting the result according to the international sensitivity index (ISI) of the thromboplastin used in the lab (Formula 1).[8] As a result of the standardization, an INR value of 1.0 is considered to be normal coagulation and an INR of 2.0 means that the time has been prolonged to double the normal time for coagulation. Untreated healthy individuals have an INR of 0.8-1.2.

[1] In the Nordic countries we use a PT test called Owren PT which measures FII, FVII and FX.

$$INR = \left(\frac{PT_{patient}}{PT_{normal}} \right)^{ISI} \qquad \textit{Formula 1}$$

INR target ranges for anticoagulation vary between countries and indications with the most common INR target range being 2.0 - 3.0 which also is the current standard for stroke prevention in AF. For patients at very high risk of thrombosis the target range can be increased to 2.5-3.5 or even higher e.g. in patients with certain types of mechanical heart valve prostheses. Patients receiving warfarin have frequent INR checks during the induction phase of therapy and once the target range for anticoagulation is achieved the INR is measured once or twice a month.

A measure of how well a patient is anticoagulated during a specific time interval is the time in therapeutic treatment range (TTR), usually INR 2.0-3.0. The standard way to calculate TTR is by the Rosendaal method, which uses linear interpolation to calculate the percent of time a patient is in treatment range.[16] TTR is associated with the efficacy of warfarin treatment where an increase of 10% in time spent outside TTR relates to an augmented risk of ischemic stroke.[17] TTR below 60 % has also been shown to affect bleeding risk, which is a common side effect of warfarin.[18]

Pharmacokinetics of warfarin

Warfarin is administered orally as a racemic mixture of R and S-enantiomers with the S-isomer being three- to five-times more potent than the R-form.[19] It is eliminated through liver metabolism and the two warfarin isomers are metabolized by different pathways. The main enzyme involved in the metabolic elimination of (S)-warfarin is CYP2C9, while (R)-warfarin is eliminated by CYP1A2/CYP3A4.[20] The average half-life of racemic warfarin is 36-42h.[1]

Starting doses

Since the time needed to reach pharmacokinetic steady state is 4 to 5 times the half-life of a drug, it would take more than a week to reach steady state if a patient starts on a maintenance dose of warfarin (*Figure 2*).

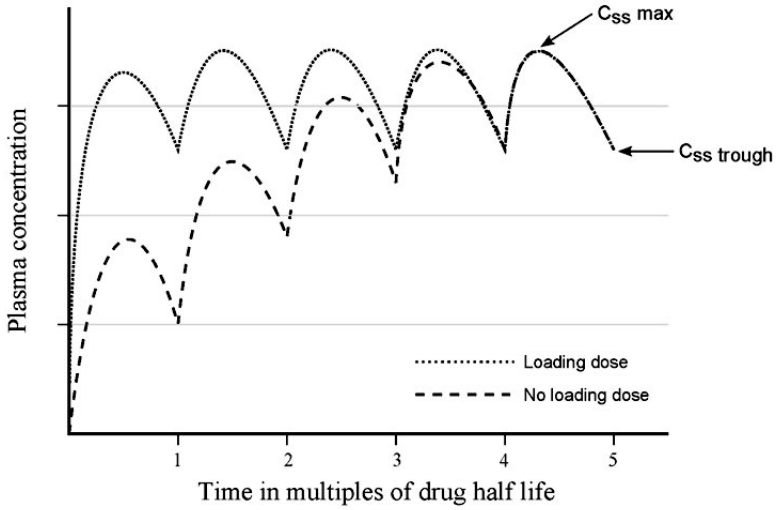

Figure 2. Oral steady state concentration-time profile. Time to reach steady state concentrations in plasma is related to the half-life of the drug. Without loading doses pharmacokinetic steady state is reached in 4-5 half times. The half-life of racemic warfarin is 36-42h. The maximum concentration at steady state is called C_{ss} max (or peak) and the minimum is called C_{ss} through.

In Sweden, it is therefore customary to initiate warfarin treatment with loading doses during the first three days. By giving a loading dose, it is theoretically possible to reach therapeutic concentrations during the first day of treatment, but, it is typically administered over two or three days.

There is no gold standard for how the induction of warfarin therapy should be performed. Some countries have a defensive strategy, starting with fairly low doses and raising the dose over time with the guidance of frequent INR tests. This method is safe with respect to over-anticoagulation, which is related to bleeding, but patients requiring higher doses than average will be under-anticoagulated for a large part of the induction phase.[21] Commonly used initiation methods include giving 5 or 10 mg on the first two days or giving age-stratified initiation doses before switching to maintenance doses.[22, 23] The Swedish strategy is offensive; an example is the loading dose regimen shown in Table 2.

Table 2. *A Swedish loading dose regimen according to www.internetmedicin.se*

Age	Day 1	Day 2	Day 3	Day 4
>85	7.5 mg	5 mg	1,25 mg	2.5 mg
76-85	7.5 mg	5 mg	3,75 mg	3,75 mg
66-75	10 mg	7.5 mg	5 mg	3,75 mg
50-65	10 mg	10 mg	7.5 mg	5 mg
15-49	10 mg	10 mg	7.5 mg	7.5 mg

With this strategy, most patients start with double their expected weekly dose of warfarin, and the INR response is checked after 2-4 doses.

Two examples of patients starting warfarin treatment are shown in *Figure 3*. There are obvious problems in reaching steady state anticoagulation within the therapeutic range for Patient B whereas Patient A reaches the target INR interval within approximately 2 weeks.

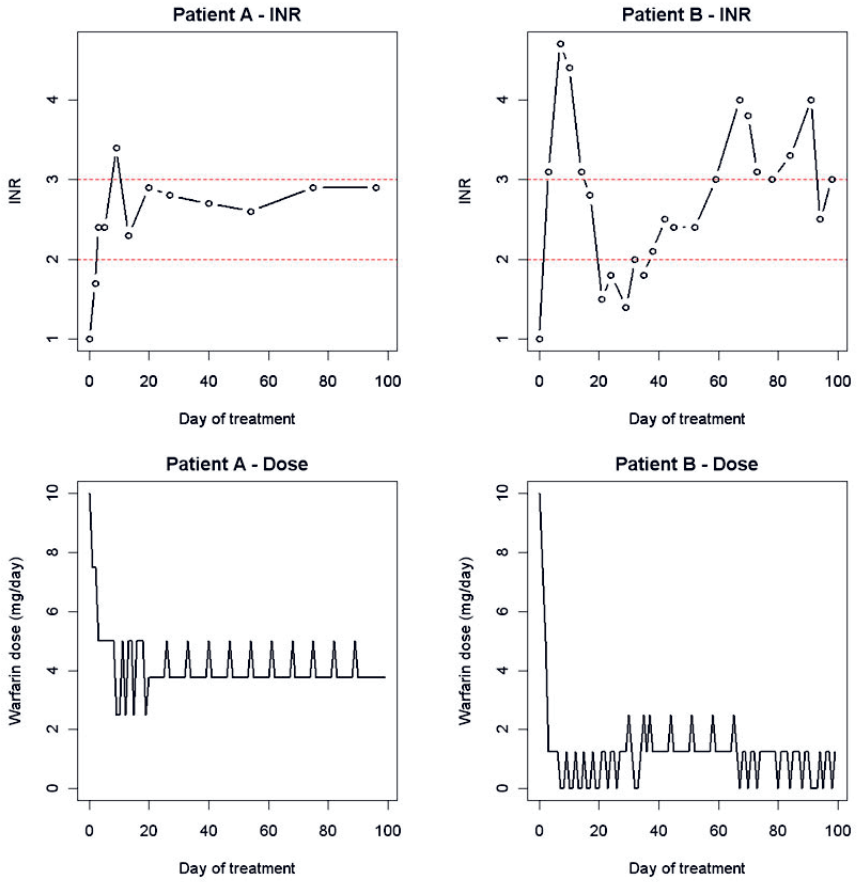

Figure 3. Example of the first 100 days of warfarin treatment for a normal dose patient (patient A) and a low dose patient (patient B). Patient A was initiated with 10 mg, 7.5 mg and 7.5 mg on day 1-3 and patient B was initiated with 10 mg, 7.5 mg and 5 mg on day 1-3. The red lines indicate the target INR range of 2.0 - 3.0

Adverse events

A common side effect of treatment with warfarin is risk of bleeding and it is related to the intensity of anticoagulation.[17] Studies have demonstrated that increasing the INR target range from 2.0 – 3.0 to 3.0 – 4.5 increases the risk

of clinically significant bleeding.[1] Although bleeding is related to the intensity of anticoagulation most bleedings occur while in therapeutic INR range of 2.0 - 3.0. These bleedings, occurring at INR levels < 3.0, are sometimes associated with trauma or an underlying diseases in the gastrointestinal or urinary tract.[1] If a patient is severely over anticoagulated with high INR levels or bleeding, treatment is normally stopped and vitamin K is administered to reverse the effects of warfarin. In case of severe bleeding there is also an option to infuse fresh plasma or prothrombin concentrate.[1]

Over the last decades, estimated risks of major bleeding on warfarin treatment have varied from 0 to 7 % per patient year.[24] Much of the variation could be due to different definitions of major bleeding being used. In 2005, the International Society on Thrombosis and Haemostasis (ISTH) published a proposal for a common definition of major bleeding.

Definition of major bleeding according to ISTH[24]

1. Fatal bleeding, and/or
2. Symptomatic bleeding in a critical area or organ, such as intracranial, intraspinal, intraocular, retroperitoneal, intra-articular or pericardial, or intramuscular with compartment syndrome, and/or
3. Bleeding causing a fall in hemoglobin level of 20g/L or more, or leading to transfusion of two or more units of whole blood or red cells.

The ISTH definition of bleeding has been used in recent trials comparing new oral anticoagulants with warfarin. The rates of major bleeding on warfarin treatment in these studies are in the range 3.1 % to 3.4 % per patient year (Table 3).[5-7] Intra cranial hemorrhage (ICH) is the most feared major bleedings, and it is often fatal.[25] The incidence of ICH in the warfarin treatment arms of the clinical trials RE-LY, ARISTOTELE and ROCKET AF were 0.74 %, 0.80 % and 0.70 % per patient year, respectively.

Table 3. *Estimates of efficacy and safety parameters from the warfarin treatment arms of recent clinical studies on patients with AF and at least one risk factor for stroke (in the ROCKET trial with at least two risk factors for stroke).*

Study	N	$CHADS_2$ (mean)	TTR (mean)	Stroke (%/year)	Major bleeding* (%/year)	ICH (%/year)
RE-LY[5]	6022	2.1	64.0 %	1.57	3.36	0.74
ARISTOTELE[6]	9081	2.1	62.2 %	1.51	3.09	0.80
ROCKET AF[7]	7133	3.46	55.0 %	2.40	3.40	0.70

* Major bleeding defined according to the ISTH criteria.[24]

The HAS-BLED risk score has recently been proposed to assess 1-year risk of major bleeding in patients with AF.[26] It assigns points for any of the following risk factors; Hypertension, Abnormal renal and liver function (1

point each), Stroke, Bleeding history, Labile INRs, Elderly and Drugs or alcohol (1 point each). Validation of the HAS-BLED score in 3665 patients treated with warfarin showed an increasing percentage of major bleeding events from 0.9 %, 3.4 %, 4.1 %, 5.8 %, 8.9 % to 9.1 % for scores from 0-5, respectively.[18] One problem with using risk-scores for major bleeding is that many of the incorporated factors are also risk factors for stroke. So far there is no recommendation on how to balance the risk of stroke versus the risk of bleeding in individual patients.

Factors affecting dose

Dietary interactions

Vitamin K is the natural antidote to warfarin. Vitamin K is found in food, and the diet therefore imposes variability in warfarin response. Normal intake of vitamin K is in the range 60-200 µg/day. Most dark green vegetables such as broccoli, brussel sprouts and spinach contain large levels of vitamin K (>100 µg/2dl) but also other common foodstuffs contain fairly large levels.[27] It is estimated that an increase in vitamin K intake of 100 µg per day for 4 consecutive days lowers the INR by 0.2.[28] Studies on combining warfarin treatment with a vitamin K supplement have been performed aiming to reduce the variability in drug response caused by dietary intake.[29] These studies show varying results but the overall conclusion is that vitamin K supplements do lower the variation in drug response caused by dietary intake. The current recommendation for patients on warfarin treatment is to keep a constant intake of vitamin K through foodstuffs to minimize variation in warfarin response.

Drug interactions

The enzymes involved in metabolic elimination of warfarin are CYP2C9 for S-warfarin and CYP1A2/CYP3A4 for R-warfarin. Patients starting or stopping drugs that are known inducers or inhibitors of these enzymes, especially CYP2C9, should have extra INR tests and their dose of warfarin adjusted. Examples of interacting drugs are amiodarone, that potentiates warfarin anticoagulation through inhibition of its metabolic clearance, and rifampicin and carbamazepine that in contrast increase its hepatic clearance.[1] Moreover antibiotics that influence the intestinal bacteria's vitamin-K production can decrease VKA dose requirements. Drugs that inhibit clotting, like aspirin, diclofenac and ibuprofen, but have no inhibiting or inducing effect on the elimination of warfarin are also regarded as interacting drugs since they increase the risk of bleeding.[1]

In general the management of interactions with warfarin of common prescription and non-prescription agents is an ongoing challenge for the health care practitioners because of limited information regarding the effects of

new drugs and herbals on the pharmacokinetics and pharmacodynamics of warfarin.[30]

Other interactions

Although warfarin is almost entirely eliminated by metabolism, recent studies show that patients with moderate and severe renal impairment (estimated glomerular filtration rate: 30–59 mL/min/1.73 m^2 and <30 mL/min/1.73 m^2) require less warfarin than those with no or mild kidney impairment.[31, 32] Among other factors that have an effect on warfarin dose are age, height, weight, ethnicity, smoking and target INR value.[33, 34]

Pharmacogenetics of warfarin

The term pharmacogenetics was first coined by a German scientist named Vogel in 1959 after several observations of hereditary drug effects.[35] It was later made clear that the activity of drug metabolizing enzymes could vary due to genetic variation. While pharmacogenetics mainly refers to inherited differences in drug metabolism, pharmacogenomics refers to the general study of all of the different genes that might determine drug response. The distinction between the terms is not quite clear and they are used interchangeably. The ultimate goal of pharmacogenetics is individualized drug therapy. What this essentially means is that the dose of a certain drug or the drug itself is chosen to optimize the effect and response in the individual. This optimization is usually done by combining genetic effects and other non-genetic effects (age, gender etc.) in a prediction model to personalize treatment for a certain individual.

Warfarin is one of the best examples where pharmacogenetics plays a major role. Part of the large variation in warfarin dose requirements was explained already in the 1990s, when the effect of variation in the *CYP2C9* gene was discovered.[36] Variation in coding parts of the *CYP2C9* gene increases the half-life of S-warfarin from 30 hours up to a maximum of about 200 hours due to changes in the amino acid sequence of the enzyme.[37] Later, the gene *VKORC1* coding for the enzyme vitamin K epoxide reductase (VKOR)[38], which is the target of warfarin, was found.[39-41] Studies on variation in this gene and its effect on warfarin dosage began to emerge in press during 2005 and they all showed that a large amount of the variability in dose requirement could be explained by polymorphisms in *VKORC1* (Paper A).[42, 43]

Genetics

The human genome consists of six billion nucleotides stored on 23 chromosome pairs. One copy of each chromosome is inherited from the mother and

one from the father. Chromosomes not linked to gender are called autosomal. The human genome has 22 autosomal chromosome pairs where the last pair differs between males and females. Females have two copies of the X chromosome while males have one X and one Y.

A chromosome is an organized structure of deoxyribonucleic acid (DNA). The structure of DNA in the form of a double helix was first published by Watson and Crick in 1953.[44] Since then the evolution in the field of genetics has been enormous. In 2001 the human genome project published the first draft of the complete human genome sequence.[45] DNA is built of two long chains of nucleotides where every nucleotide includes one of the bases adenine (A), thymine (T), guanine (G) and cytosine (C). When forming the structure of DNA, A always pairs with T and C always pairs with G. This is one of the key factors of DNA replication.[46]

Genes and genetic variation

A gene is a functional unit of DNA which codes for a certain protein or functional ribonucleic acid (RNA) molecule. The definition of a gene also includes sequences that regulate gene activity. These sequences may be close to or distant from the coding parts. In 2004 an updated report from the human genome project estimated that the human genome consists of 20.000 to 25.000 protein coding genes.[47]

There are a number of steps involved in the process of creating a protein from a gene; in short the steps are transcription, splicing and translation. During transcription the DNA information in a gene is copied into RNA. The next step is splicing; here the introns are cut out of the RNA and the exons are merged to form messenger RNA (mRNA), thus the exons of a gene are the parts that code for proteins. The next step is translation when the transcribed mRNA is translated into a protein. In some genes the RNA transcript is functional without being translated to a protein. There are different types of functional RNA and the main classes contribute to the process of translating mRNA to protein.

Except for monozygotic twins, every human has a unique genome. However, the variation between genomes is quite small, and two genomes are roughly 99.9% identical.[48] The small fraction that differs is the cause of genetic heritability among individuals and may play a part in the susceptibility to diseases and drug response. The most common form of genetic variability is the single nucleotide polymorphism (SNP). Examples of other sources of variation are insertion/deletions, copy number variations and simple sequence length polymorphisms. As of 5th April 2012 there are 50 million SNPs and 8 million insertions or deletions of DNA sequence registered for the human genome in the National Center for Biotechnology Information (NCBI) SNP database (dbSNP build 135).

A SNP is a position in the DNA where a single nucleotide has been substituted for another. For example, comparing the strings TG**T**TA and TG**C**-TA in *Figure 4* will reveal that a T has been changed to a C. This means that we have two different variants (alleles). The least common is called the minor allele and the most common is called the wild type allele. The frequency of a SNP is measured by the minor allele frequency (MAF). A person carrying the alleles in *Figure 4* would be heterozygous C/T. The other possible genotypes are homozygous for any of the two alleles (i.e. C/C or T/T). The genetic position of a SNP could be anywhere in the human DNA, within a gene or outside a gene. If the SNP is in a coding part of a gene (exon) and changes the amino acid sequence that is produced it is called a non-synonymous SNP. If the SNP is in an exon but does not change the protein it is called synonymous. However, even synonymous SNPs and non-coding SNPs may affect the expression or function of a protein, i.e. be functional SNPs.

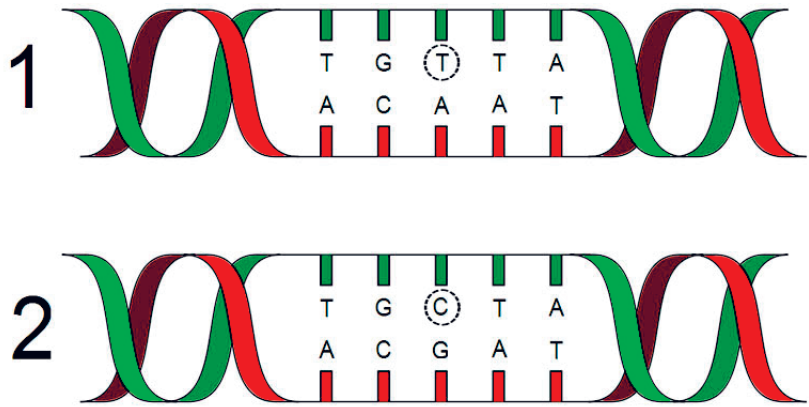

Figure 4. Example of a SNP. The figure shows two pieces of DNA from a chromosome pair, 1 is the first chromosome and 2 is the second chromosome. T in the first DNA sequence has been changed to a C in the second DNA sequence. The SNP has the alleles C/T

Genetic association studies

The goal of association studies in genetics and pharmacogenetics is to link a phenotype, e.g. disease, dose of drug or adverse event, to genetic variation. The most commonly used genetic variants in these studies today are SNPs.

Linkage disequilibrium

There is often an amount of correlation between SNPs in the same chromosomal region. In genetics the amount of dependency between two SNPs is called linkage disequilibrium (LD). The most common measures of LD are

D, D' and r^2 where D' and r^2 are standardized versions of D. The measures have different properties and may be used for different purposes. D' is useful for assessing historical recombination while r^2 is useful in association studies.[49] From a statistical view r^2 is the most intuitive measure since it can be interpreted as the squared correlation between SNPs where a value of 1 would mean that they are in total LD (always inherited together) and 0 would mean that they are totally independent.

Haplotypes

Chromosomes in human cells occur in pairs and one chromosome is inherited from each ancestor. However the chromosomes are not passed on as identical copies of the original ones. They are slightly changed in a process called recombination which takes place when egg cells and sperm are formed. During recombination the two chromosomes in a pair exchange pieces of DNA which results in a new chromosome pair containing parts from both chromosomes.

A haplotype or haplotype block is a region of high LD where the frequency of recombination has been low throughout history. The haplotype blocks are separated by places where recombination has occurred, these places are called recombination hot-spots. Often the variation in a haplotype block is defined by one SNP; this SNP is called a haplotype tagging SNP or tag SNP. Haplotype blocks may include many SNPs; however the easiest way to describe a haplotype is by illustrating a haplotype only consisting of two SNPs.

Table 4. *Example of haplotype frequencies in a 2x2 table*

		SNP 2		
	Alleles	A	G	
SNP 1	C	n_{11}	n_{12}	n_{1+}
	T	n_{21}	n_{22}	n_{2+}
		n_{+1}	n_{+2}	n

In Table 4 a 2x2 table is shown with the haplotype frequencies of two SNPs. Four possible haplotypes can be inherited from two SNPs and in the example they are CA, CG, TA and TG. In the table n_{11} is the number of haplotypes CA, n_{1+} is the total number of haplotypes with a C allele, n_{+1} is the total number of haplotypes with a A allele and n is the total number of haplotypes. Although there is only one SNP shown in *Figure 4*, the haplotypes (including all alleles) are TG**T**TA and TG**C**TA. If there would be a SNP in the first position also changing the T to a C, the four possible haplotype combinations would be **T**G**T**TA, **T**G**C**TA, **C**G**T**TA and **C**G**C**TA.

Haplotype analyses has the potential to increase the statistical power compared to single marker analyses in association analyses by capturing the available LD information within a region.[50]

Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium (HWE) is named after the two people who independently discovered the association between allele frequencies and expected genotype frequencies. When a SNP is in HWE the genotype frequencies are constant in successive generations within a population assuming random mating. The expected genotype frequencies for a SNP under HWE are calculated using the MAF and are shown in *Figure 5*.

There are a couple of reasons why a SNP could be out of HWE. For example the SNP could be selected for or against, or the allele frequency could change through a random process (genetic drift). In addition, the cause could be genotyping errors, and checking a marker for HWE as a quality control is common practice in genetic studies. It is simply done by comparing the observed frequencies with expected frequencies under HWE assumptions using a χ^2 test with one degree of freedom (= number of genotypes – number of alleles).

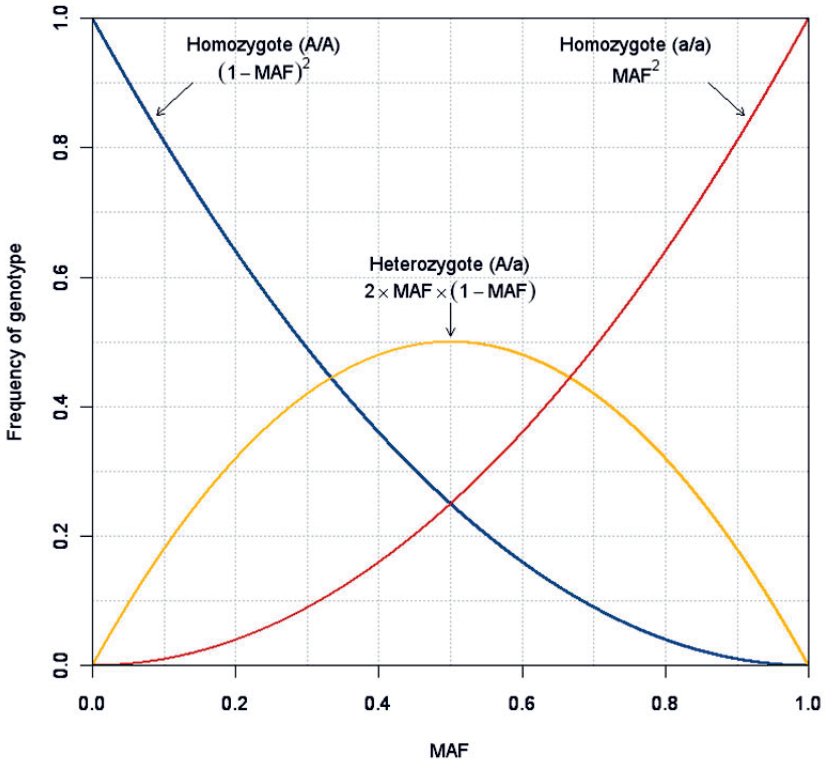

Figure 5. Expected genotype frequencies by MAF (allele frequency of allele "a") for SNPs that are in HWE. Note that when passing MAF of 0.5 there is a switch in minor allele

Genome-wide association studies

Lately new biotechnological tools have moved the focus of many genetic studies from candidate genes to a genome-wide approach. These genome-wide association studies (GWAS) enable the simultaneous association of >1 million SNPs with different outcomes. A large proportion of the SNPs in the first genome-wide chips by Illumina were tag SNPs taken from the international HapMap Project.[51] By selecting only tag SNPs, variation in the genome can be analyzed using a minimal number of SNPs.

Apart from using the LD pattern in the human genome to select SNPs to be included in genome-wide genotyping arrays, the LD can be used for imputation of untyped SNPs in association studies. Imputations can be done either to fill in missing genotypes for a marker or to impute new markers in data.[52] Imputing SNPs is the key factor when combining studies, performed on different arrays, for meta-analysis. When imputing new SNPs, publicly available reference sets are used. Today there are two major projects providing this type of data, the HapMap project and the 1000 Genomes Project.[51, 53] HapMap (release 3) has sequenced over 1000 individuals and the 1000 Genomes Project aims to sequence 2500 individuals.[54] A number of computer software programs have been developed utilizing different algorithms and approaches to imputation on a genome-wide scale. The most common programs are Impute2, MaCH and Beagle.[55-57]

Population stratification is a problem that could arise in genetic studies, typically in genome-wide studies including data from different ethnic populations or countries. Population stratification could for example arise when the mean of a continuous phenotype varies among ethnic subpopulations of a study; in this case, SNPs that have no mechanistic connection to the phenotype but differ in allele frequencies among subpopulations will be associated to the phenotype.[58] The effect of stratification could be reduced power and increased risk of spurious findings.[59] Different methods for handling population stratification have been presented; these include genomic control using the inflation factor (λ), structured analysis, principal component analysis[58] and the EIGENSTRAT method.[59]

Multiplicity

The number of statistical tests involved in candidate gene studies and whole genome studies has made the risk of false positive results (= the type I error) into a major problem. This has made it necessary to verify results by replication in separate cohorts. Correcting for the number of statistical tests performed is the first step towards keeping the type I error at a nominal level (e.g. 5%) thus lowering the risk of spurious results. Different methods are used to handle this problem in genetic studies; the "gold standard" would be permutation tests, but when dealing with whole genome studies many researchers use the conservative Bonferroni method. The Bonferroni method

basically means that the significance level of each test is set to α/number of tests (where α usually is set to 0.05). This method is conservative and assumes that the tests performed are independent of each other. In genetic studies where multiple SNPs, often within the same gene, are compared the assumption of independence does not hold. For instance comparing two SNPs that are in high LD means that almost the same test is done twice and Bonferroni correction would be too conservative. A method that adjusts the significance level based on the LD among markers is called the M_{eff} method.[60, 61] With this method the effective number of tests is calculated. The formula is given in Formula 2 and it requires two arguments, one is the total number of SNPs tested (M) and the other is the variance of the calculated eigenvalues $Var(\lambda_{obs})$.

$$M_{eff} = 1 + (M - 1)\left(1 - \frac{Var(\lambda_{obs})}{M}\right) \qquad \textit{Formula 2}$$

The eigenvalues are a result of principal component analysis on the LD matrix between SNPs. The result gives the total number of effective tests, M_{eff}, which can be used as the number of tests done when applying Bonferroni correction. In short, if one SNP explains all variability in the LD matrix (all SNPs are inherited together) then $Var(\lambda_{obs})$ will be equal to M and the equation will result in 1 test performed. If all SNPs are totally independent the opposite will happen, $Var(\lambda_{obs})$ will be 0 and the result will be that M tests have been performed. Li MX. et al. made an effort to extend the M_{eff} method to evaluate the effective number of tests in genome-wide scans.[62] When comparing the total number of tests (561716 = number of SNPs) using the Illumina 610quad chip, they estimated the total number of effective tests to be 374316. The effective ratio is 0.666 i.e. using the M_{eff} method adjusts for 33.4% less tests.

Among other methods that are used to adjust for multiplicity are the false discovery rate and the qvalue method.[63]

Prediction models

A prediction model is an algorithm relating a certain measure or measures to an outcome. In the field of medicine, it is most commonly the result of a regression model with estimated coefficients as weights of each variable.

It is important that the data a prediction model is built upon reflects the population that it will be predicted on. For instance, if we want to build a prediction model that includes age, the data used to build the model should include the age that we will be predicting in. If we built a model on data with ages 20 to 40 years and predicted on patients aged 60 to 80, there is a chance

that the coefficient for age is wrong, the age-outcome relationship might for instance be of second order. Narrow intervals or selected populations are often the case in phase I-II clinical trials, which could make them unsuitable for use as base for deriving prediction models.

Least squares regression

The least squares method was first published in 1805 by the French mathematician Legendre. Some years later a German mathematician Carl Friedrich Gauss published the same method claiming he had been using it since 1795.

Nowadays modern statistical computer packages and spread sheet programs estimate the coefficients of simple and multiple regression models in a fraction of a second. It is however of importance to know the basics of regression to be able to interpret the result of a regression model. One key to understanding the results is to be able to write down the model that is being used. The simplest linear regression model could be expressed as

$$y_i = \beta_0 + \beta_1 \mathrm{x}_\mathrm{i} + \varepsilon_\mathrm{i} \qquad \textit{Formula 3}$$

where $i=1,\ldots,n$ and n is the number of observations. The model in Formula 3 is most commonly referred to as a simple, univariate or univariable model because it has only one independent variable x. The parameters on the right hand side are β_0 which is called the intercept, β_1 which is the coefficient or slope for x. ε_i is called the residual or error term. A model with more than one independent variable is written as

$$y_i = \beta_0 + \beta_1 \mathrm{x}_{\mathrm{i}1} + \beta_2 \mathrm{x}_{\mathrm{i}2} + \ldots + \beta_\mathrm{k} \mathrm{x}_{\mathrm{ik}} + \varepsilon_\mathrm{i} \qquad \textit{Formula 4}$$

and is called a multiple regression model. Here k is the number of independent variables in the model.

In least squares regression, the model fit is maximized by minimizing the sums of squares (SS) of the residuals. For simple regression this is given by S in Formula 5.

$$S = \sum_i \varepsilon_\mathrm{i}^{\,2} = \sum_i (y_i - \beta_0 - \beta_1 \mathrm{x}_\mathrm{i})^2 \qquad \textit{Formula 5}$$

To estimate the coefficients $b_0 = \beta_0$ and $b_1 = \beta_1$ the partial derivatives of S with respect to β_0 and β_1 are set to 0 and solved. The resulting estimates b_0 and b_1 are shown below in Formula 6.

$$b_0 = \bar{y} - b_1 \bar{x}$$
$$b_1 = \frac{\sum_i (y_i - \bar{y})(x_i - \bar{x})}{\sum_i (x_i - \bar{x})^2} \qquad \textit{Formula 6}$$

From b_0 and b_1 the predicted values $\hat{y}_i$ are calculated as $\hat{y}_i = b_0 + b_1 x_i$.

Variable selection

Usually researchers start out with a fairly long list of independent variables that are a combination of new potential predictors of the outcome and previously known and validated predictors of the outcome. A prediction model could simply be estimated including all independent variables in a multiple regression model. However, most often we wish to delete certain variables from the model because it is easier to work with simpler models. Deleting variables might also have impact on the clinical cost of using the model, e.g. if a SNP or an expensive biomarker is deleted from the model the cost of using it will be lowered.

In regression, decisions to delete variables could be made by examining the estimated beta coefficients and their standard errors. Variable deletion could also be performed by investigating relationships between the independent variables and dropping variables that are redundant (i.e. two variables that are highly correlated). However, the ways to decide which variables that should be deleted are many and the model chosen as the final model often reflects the analyst's best judgment at the time of the modeling process.

Measures and plots of model performance

A common measure of the performance of a linear regression model is given by the coefficient of determination (R^2). R^2 is a measure of how much of the variation in the outcome (y) that can be explained by a model (Formula 7).

$$R^2 = 1 - \frac{SS_{err}}{SS_{tot}} \qquad \textit{Formula 7}$$

To calculate the R^2, the SS for error (SS_{err}) and the SS for total (SS_{tot}), that are given in equation Formula 8, are needed.

$$SS_{err} = \sum_i (y_i - \hat{y}_i)^2$$

$$SS_{tot} = \sum_i (y_i - \bar{y})^2 \qquad \textit{Formula 8}$$

$$\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i \text{ (overall mean of y)}$$

$$\hat{y}_i = \text{predicted value for patient i}$$

From equations Formula 7 and Formula 8 we see that a prediction model that results in perfect prediction gives an SS_{err} of 0 and an R^2 of 1, SS_{err} closer to the total variation SS_{tot} yields an R^2 closer to 0. R^2 can also be calculated by squaring the correlation, R, between the true observed values and the predicted values from a prediction model.

Although estimations of R^2 are informative in terms of model performance, they are rather abstract. A more intuitive measure is the mean absolute error (MAE). The formula for MAE is given in Formula 9.

$$MAE = \frac{1}{n}\sum_i |y_i - \hat{y}_i| \qquad \textit{Formula 9}$$

The interpretation of MAE is more straightforward than R^2 mainly because it can be reported on the original scale of the outcome. If the outcome is dose then a MAE of 8 mg/week would mean that the average patient will be predicted within 8 mg/week of the true value.

R^2 and MAE are good measures of model performance; however, they do not give the "whole" picture of how a prediction model performs. A common way to illustrate the performance of a prediction model is by plotting the observed versus the predicted values (*Figure 6* under results on page 48). This type of calibration plot gives information on how well the model performs across the range of available observed values.

Validation of prediction models

Developing and evaluating prediction models on the same data can result in optimistic measures of model performance. This is caused by over fitting the model to the data which results in a positive bias on model performance (e.g. over optimistic R^2 values).

External validation

In terms of model validation, there are two paths one can choose; external validation and internal validation. The optimal way to generate unbiased estimates of model performance is to use external validation. Here we apply

our prediction model on a data set, including patients independent of the modeling process.

Differences in model performance between derivation data set and external validation data set could be many. For instance, compared to the derivation data set the external validation data set could have different definitions on the dependent and independent variables and other mix of patient characteristics. For "real life" estimates of performance the external validation data set should resemble the population we will be using the prediction model on.

Internal validation

If no external validation set is available and the data set at hand is fairly large it can be divided into a derivation data set, where the model parameters are estimated, and a validation data set, where the model performance is estimated. The split into derivation and validation data sets should be done before any formal inference is done on the data since this has potential to bias the model performance in the validation data set. A result of data-splitting is reduced sample size for both model development and model testing. However, after validation the final model parameters can be estimated in all data for increased precision in beta estimates.[64]

Other methods for internal validation include resampling techniques such as bootstrapping, cross-validation and jackknifing. These methods can be used to obtain nearly unbiased estimates of model performance but in this case they require that the variable selection process is fully automated.[64] In general the methods involve a few steps:

1. Randomly sample the data into derivation and validation data sets (with or without replacement)
2. Derive model parameters on the derivation data
3. Estimate performance on the validation data (e.g. R^2)

The steps above are repeated a number of times (depending on the method) and the resulting distribution of the performance parameter is summarized.

The resampling methods have different strengths and weaknesses, both in the estimation of model performance and in the interpretation and translation to a clinical public. Therefore, as with variable selection, the choice of method often reflects the analyst's best judgment.

Aim of the thesis

The studies presented aim to evaluate mainly genetic factors, but also non-genetic, that affect the response to warfarin in terms of required maintenance dose, efficacy and safety with special focus on warfarin dose prediction.

Specific aims were:

Paper I

- Determine which candidate genes that have effects on warfarin dose, time to stable anticoagulation, time to first INR peak > 4.0 and TTR.
- Evaluate if the risk of bleeding is related to variants that affect the outcomes above.
- Estimate a prediction model for warfarin maintenance dose.

Paper II

- Estimate a prediction model for mean warfarin dose based on a multi-ethnic pooled dataset and evaluate its clinical usefulness.

Paper III

- Assess the influence of common SNPs in *VKORC1* on warfarin dose among Asians, blacks and whites.
- Evaluate whether other *VKORC1* SNPs and haplotypes explain additional variation in warfarin dose in each ethnic group, beyond the *VKORC1* rs9923231 SNP included in the IWPC prediction model derived in Paper II.

Paper IV

- Analyze the whole genome for variants affecting clinical outcomes, warfarin maintenance dose and TTR.
- Estimate the performance of the IWPC model derived in Paper II.
- Evaluate if renal function (CrCl), smoking or *CYP4F2* rs2108622 should be added to future prediction models for warfarin dose.

Paper V

- Provide a broad review of the field of warfarin pharmacogenetics with special focus on prediction models and how they can be used.

Materials and methods

Paper V is a review paper and it is therefore only mentioned in the Results and Discussion.

Subjects

Paper I

The WARG study was a prospective multicenter study of warfarin bleeding complications and predictors of response to warfarin.[65] Between 2001 and 2005, patients starting warfarin were collected at 40 outpatient clinics distributed throughout Sweden. The majority of these centers were specialized at anticoagulation; the remainders were primary health care centers. The patients were subject to treatment according to standard care at each center, without specific warfarin dosing algorithms. In total, 1523 first-time warfarin users aged 18 to 92 years were recruited. The majority was of Swedish origin, but ethnicity was not registered. Patients were recruited regardless of indication for treatment, and apart from established contraindications for warfarin, the only exclusion criteria were previous exposure to warfarin and age younger than 18 years. There were no restrictions regarding target INR, planned treatment duration or comorbidities.

One hundred eighty-one previously genotyped patients from the pilot cohort (used in Paper A and Paper B, called the Uppsala study) were used to validate the algorithm derived on the WARG cohort. In the pilot cohort, included patients had been treated with warfarin for at least 2 months. They were enrolled at the Uppsala University Hospital during 2000. The primary aim was to study factors that influence warfarin dose.

Paper II

Subjects used in paper II were assembled by the International Warfarin Pharmacogenetics Consortium (IWPC). The consortium included 21 research groups from 9 countries and 4 continents. The result was a database of 5700 warfarin treated patients. The contribution from Sweden was the Uppsala study, partly used in paper I. The cohort whose data were analyzed for this study included the subgroup of 5052 patients who had a target INR of 2.0 to 3.0.

Paper III

The subjects from the initial IWPC paper (Paper II) were extended with 556 additional patients (20 Asians, 230 African Americans, 302 white and 4 of unreported ethnicity) to a total of 6256 warfarin treated patients. The motive was mainly to raise the number of African American patients to enable better comparisons between ethnicities.

Further, a worldwide haplotype survey including 6 *VKORC1* SNPs on 8751 subjects was created using 1306 subjects from the IWPC cohort (138 with genotype information only); 317 subjects from 6 different Asian countries with genotype information only; 316 subjects from 5 countries in South America , Africa, and the Middle East with genotype information only who were recruited as part of the PharmacoGenetics for Every Nation Initiative; and 6812 participants (2108 non-Hispanic blacks, 2631 non-Hispanic whites, and 2073 Mexican Americans) ascertained as part of the Third National Health and Nutrition Examination Survey (NHANES), a population-based, racially representative cross-sectional study in the United States.

Paper IV

RE-LY was a randomized trial comparing two doses of dabigatran (110mg or 150mg twice daily) with warfarin for stroke prevention in 18,113 patients with documented AF and at least one additional risk factor for stroke. The primary efficacy endpoint of the RE-LY trial was stroke or systemic embolism and the primary safety outcome was major bleeding during a mean follow-up of 2 years. The study design and results have been described previously.[5, 12] In the genomic substudy of the RE-LY trial, 3,076 patients consented to provide blood samples for DNA analyses. Paper IV focuses on the 982 patients with genetic samples in the warfarin treatment arm.

Eligibility for the trial required documented atrial fibrillation and at least one of the following additional risk factors; (1) History of previous stroke, TIA or systemic embolism; (2) Ejection fraction < 0.40; (3) Symptomatic heart failure, New York Heart Association class 2 or higher in the last 6 months; (4) Age at least 75 years or age at least 65 years and any of diabetes mellitus, hypertension or coronary artery disease.

Among the exclusion criteria were severe heart valve disorder, recent stroke, increased risk of haemorrhage, creatinine clearance less than 30 mL/min, or active liver disease.

Patients on VKA treatment at the time of randomization stopped their treatment on the day of randomization and began the randomized treatment at INR below 2.0 if assigned to dabigatran and below 3.0 if assigned to warfarin. For patients on warfarin, the local investigator was responsible for the dose adjustments required to reach and maintain an INR of 2.0-3.0. After the initial dose titration phase patients randomized to warfarin underwent INR testing at least once a month. The TTR was closely monitored and measures

were adopted to maximize the TTR.[12] A dose-adjustment algorithm was provided, but its usage was not mandatory according to the study protocol.[12] The dose-adjustment algorithm included an initiation nomogram and an action table, proposing dose adjustments based on changes of INR levels obtained after initiation or alterations of the warfarin dose.

Outcome measurements

Paper I

Warfarin maintenance dose was calculated as the mean of all weekly doses associated with stable anticoagulation periods. A stable anticoagulation period was defined as a period of at least three INR measures in the range of 2.0 – 3.0. A second definition of warfarin maintenance dose was also used; here maintenance dose was defined as the mean of all weekly doses that are unchanged over a period of at least three consecutive visits during stable anticoagulation periods. Major bleeding was defined according to the World Health Organization (WHO) criteria for a serious adverse drug reaction, that is, if it was lethal, life-threatening, permanently disabling, or lead to hospital admission or prolongation of hospital stay. Time to stable anticoagulation was defined as the time to the first INR in a stable anticoagulation period. TTR (called TIR in Paper I) was calculated using linear interpolation according to the Rosendaal method.[16]

Paper II and Paper III

Warfarin maintenance dose was calculated using a wide range of definitions selected by the participating groups. Although the centers used different definitions for steady-state dose, most centers required stable levels of anticoagulation (i.e., INR) over a period during which the dose of warfarin was stable. The information is available in the online supplementary appendix section S2 to Paper II.

Paper IV

Warfarin maintenance dose was calculated as the mean of all doses during stable anticoagulation periods. A stable anticoagulation period was defined as a period of at least three measures of INR within 2.0-3.0. In patients where no stable anticoagulation periods were observed, the maintenance dose was calculated as the mean of all doses associated with an INR between 2.0 and 3.0. TTR for the first 3 months (TTR 3M) and TTR during the whole treatment period (TTR Total) were calculated using linear interpolation according to the Rosendaal method.[16] TTR 3M was calculated using all values regardless of treatment initiation or interruption and TTR Total was calculated excluding the first seven days of treatment and treatment interruptions.

Stroke was defined as the sudden onset of a focal neurologic deficit in a location consistent with the territory of a major cerebral artery.[5] Systemic embolism was defined as an acute vascular occlusion of an extremity or organ, documented by means of imaging, surgery, or autopsy.[5] Major bleeding was defined as a reduction in haemoglobin concentration by at least 20 g/L, transfusion of at least two units of blood, or symptomatic bleeding in a crucial area or organ.[5] Creatinine clearance (CrCL [mL/min]) was calculated according to the Cockroft-Gault formula.[5]

Statistical methods

Paper I

Univariate and multivariable analyses of predictor impact on the square root of warfarin dose and TTR were calculated using linear regression analyses.

Association with time to stable INR, over-anticoagulation, and bleeding were evaluated with the logrank test. Hazard ratios were estimated with Cox regression analyses. Risk of bleeding was compared with the Fisher exact test and the Pearson χ^2 test.

The prediction models were based on verified findings and only significant variables ($P < 0.05$) were allowed in the final model. Performance of the final prediction model was evaluated with cross validation to achieve less biased estimates of R^2. The training data set was randomly selected as 70% of the data and the procedure was repeated 10 000 times. The median and 2.5/97.5 percentiles of the resulting distribution of the R^2 values were calculated as estimate and 95 % confidence interval, respectively. The final prediction model was validated in a separate cohort.

Paper II

We randomly chose 80 % of the eligible patients, (stratified according to site, for a total of 4043 patients who had a stable dose of warfarin and a target INR of 2.0 to 3.0) as the "derivation cohort" for developing all dose-prediction models. The remaining 20 % of the patients (1009 patients, from all 21 sites) constituted the "validation cohort," which was used for testing the final selected model. The investigators who performed the modeling and analysis did not have access to this validation set until after the final model was selected. A wide variety of numerical modeling methods were used for the data from the derivation cohort, including, but not limited to, support vector regression, regression trees, model trees, multivariate adaptive regression splines, least-angle regression, and Lasso, in addition to ordinary linear regression. Logarithmic and square root transformations of doses were tested, in addition to a direct prediction of dose. MAE was used to evaluate each model's predictive accuracy. The MAE was reported on the original scale of

warfarin dose. We selected the final model as the one that had the lowest predictive MAE as estimated by 10-fold cross validation on the derivation cohort.

Using the validation data set, we compared dose predictions from the pharmacogenetic model with those from two other models: a clinical model that did not include genetic factors and a model with a fixed dose of 5 mg of warfarin per day. The clinical model was built with the use of the same methods as the pharmacogenetic model, but without the incorporation of genetic variables. MAE and R^2 in the validation data set were our prespecified metrics for evaluating the pharmacogenetic, clinical, and fixed-dose models. We evaluated the potential clinical value of each algorithm by calculating the percentage of patients whose predicted dose of warfarin was within 20% of the actual stable therapeutic dose. In addition, we calculated the percentage of patients for whom the predicted dose according to each algorithm was at least 20% higher than the actual dose (overestimation) or at least 20% lower than the actual dose (underestimation). These values represent a difference of 1 mg per day relative to the traditional starting dose of 5 mg per day, a difference clinicians would be likely to define as clinically relevant. We also assessed the performance of the algorithms in three dose groups: participants requiring a low dose (≤21 mg per week), those requiring a high dose (≥49 mg per week), and those requiring intermediate doses (>21 and <49 mg per week) for stable therapeutic anticoagulation. These thresholds of 21 mg and 49 mg per week bracket the usual starting dose of 35 mg per week (5 mg per day). Patients requiring doses of less than 21 mg per week would be at risk for excessive anticoagulation if they were started on the standard dose of 35 mg per week. Conversely, patients requiring doses of more than 49 mg per week would be at risk for inadequate anticoagulation if they were started on a dose of 35 mg per week. We assessed the potential benefit of using the pharmacogenetic algorithm instead of the clinical algorithm or the fixed-dose model and computed the number needed to genotype (NNG, i.e., the number of patients who must be genotyped in order for one patient to have an improved dose estimate). Finally, in a post hoc analysis, we assessed how well the algorithms predicted which patients actually required low or high doses.

Paper III

Differences in allele frequencies and clinical characteristics among racial groups were compared with Fisher's Exact Test or Kruskal-Wallis test for categorical and continuous variables, respectively.

Missing values of height and weight were imputed with best-subsets regression, which determines the best fit (highest R^2) multiple regression model based on the predictor variables used for the imputation. For the height variable, weight, race, and sex were used for the imputation. For the weight variable, height, race, and sex were used.

For all analyses, the outcome variable warfarin dose (in mg per week) was square root–transformed to attain normality. Evaluation of the effects of individual *VKORC1* SNPs and haplotypes on warfarin dose used both univariate and multivariable linear regressions. Covariates included in multivariable linear regression were the same as those included in the IWPC pharmacogenetic dosing algorithm: *CYP2C9* *2 and *3, age, height, weight, and concurrent use of *CYP2C9* enzyme inducers and amiodarone (Paper II).

Because the analyses reported here were done in race-stratified subgroups of the expanded IWPC cohort, the race variables included in the IWPC algorithm were rendered uninformative and were not used. For some analyses, the IWPC algorithm was used to generate warfarin dose predictions and residuals for the patients in the expanded IWPC cohort.

A significance level α of 0.05 was used except for the residual analyses (to identify additional SNP and haplotype effects) where a more stringent α of 0.0025 was used to control for type I error.

Paper IV

Genome-wide analyses

Genome-wide analyses of all outcomes were done assuming an additive genetic model i.e. the genotypes for each SNP were coded 0, 1 and 2 and handled as continuous variables. To account for the amount of multiplicity a p-value threshold of genome-wide significance was set at $0.05/554{,}725 \approx 9 \times 10^{-8}$ according to the Bonferroni method.

GWAS of warfarin maintenance dose was performed using linear regression of square root of warfarin dose (transformed to obtain normality). Two models were run which included either the non-genetic clinical variables from the IWPC model or the full set of IWPC variables (Paper II). The full IWPC pharmacogenetic prediction model for warfarin maintenance dose includes the following variables; age, height, weight, race, enzyme inducer status (taking carbamazepine, phenytoin or rifampicin), amiodarone, *VKORC1* rs9923231 and *CYP2C9* *2/*3. Our first model included age, weight (kg), height (cm), amiodarone under treatment and the first four genetic principal components to correct for population stratification. Our second model included the same covariates as the first model with the addition of *VKORC1* rs9923231 and *CYP2C9* *2/*3.

When analyzing the outcomes TTR during the first three months, total TTR, stroke or systemic embolism and major bleeding, selection of variables for adjustment of the GWAS analyses was done as follows. First, effects on the outcomes were estimated. Variables with univariate $p < 0.10$ were analyzed in a multiple model and selected if $p < 0.10$ in the multiple model. Variables with previously known clinical effect could be added to the final adjustment variables although $p \geq 0.10$ based on judgment of beta estimates and precision. Assumptions of linear effect of continuous variables were

inspected and where nonlinear effects were present appropriate transformation was made. The variables eligible for inclusion as covariates were; age, weight [kg], height [cm], BMI [kg/m^2], gender, systolic and diastolic blood pressure at baseline [mmHg], CrCl [mL/min], type of AF, $CHADS_2$ score[3], previous stroke, previous diabetes mellitus, previous hypertension, history of VKA use, VKA use class at entry, baseline INR, treatment with aspirin, angiotensin-II receptor antagonists, ACE-inhibitors, clopidogrel, amiodarone, statins, proton pump inhibitors, H2-blockers, P-gp inhibitors and any inducer status (patients taking any of carbamazepine, phenytoin or rifampicin).

Linear regression was used for the GWAS of TTR during the first three months and total TTR, Cox regression was used for the GWAS of stroke or systemic embolism and major bleeding.

All statistical analyses on a genome-wide scale were performed using GenABEL.[66]

Analysis of phenotype and candidate gene data

Univariate effects of *VKORC1* and *CYP2C9* on TTR outcomes were evaluated using the nonparametric Kruskal-Wallis test (due to the skewed distribution of TTR INR below 2.0 and TTR INR above 3.0). Performance of the IWPC prediction model was evaluated with R^2 and MAE. Further the clinical usefulness was evaluated, as in the IWPC paper (Paper II), by calculating the percentage of patients predicted within 20 % of the observed dose in total and in subclasses of patients with observed dose $\leq$ 21 mg/week, $>$ 21 mg/week to $<$ 49 mg/week and $\geq$ 49 mg/week. Univariate and multiple analyses of candidate phenotypes and genes with potential to be included in future prediction models were done using linear regression on the square root transformed dose. Univariate R^2 values were reported on the original scale of the variable by retransforming and calculating the squared correlation between the predicted and observed values. The added R^2, to the pharmacogenetic base model included the IWPC covariates explained earlier, was evaluated using cross validation with 10 000 resamples of data; 70 % as training and 30 % as validation. The median and percentiles 2.5 and 97.5 of the resulting R^2 distribution (retransformed) were presented as point estimate and 95 % confidence interval. As with the IWPC prediction model results, the candidate phenotypes and genes were evaluated by calculating the percentage of patients predicted within 20 % of the observed dose in total and in subclasses of patients with observed dose $\leq$ 21 mg/week, $>$ 21 mg/week to $<$ 49 mg/week and $\geq$49 mg/week. The number needed to treat (NNT), to prevent one patient from being predicted 20 % from the actual dose, was also calculated.[67] To graphically evaluate additional factors, we plotted the difference in absolute residual of the base model with a new model that included the additional covariates. The reported values of difference are on the original scale of the variable. A negative value indicates that the prediction is

worse with the new model and a positive value indicates that the prediction is better.

In the analyses of phenotype and candidate gene data the significance level was set at 0.05.

Statistical-genetics

Paper I

LD was visualized using the HaploView software.[68] The QTPHASE component of UNPHASED software was used to test for association of haplotypes with warfarin dose.[69] Pair-wise LD was quantified by the standard r^2 measure.[49] The coefficient of determination, R^2, was used to measure the proportion of explained variance. To account for partial dependence among tests of SNPs in LD, we applied a Bonferroni correction for multiple testing based on the effective number of independent tests (M_{eff}) calculated by a spectral decomposition method. [60, 61] M_{eff} was 172 in our study and hence a P value of less than 2.9×10^{-4} was required for statistical significance.

Paper II

Missing values for the *VKORC1* SNP rs9923231 were imputed on the basis of race and on the basis of LD between the other *VKORC1* SNPs rs2359612, rs9934438 and rs8050894. If the VKORC1 genotype could not be imputed, it was treated as "missing" (a distinct variable level) in the model.

Paper III

Deviation from HWE was evaluated separately for each SNP within each racial group using the χ^2 test. Values for LD between all pairs of *VKORC1* SNPs were calculated with Haploview[68] and expressed as r^2.[49]

VKORC1 haplotypes were inferred with the use of SAS Genetics (SAS Institute). Because different contributing sites genotyped different numbers of *VKORC1* SNPs in their patients, the IWPC dataset contained a substantial proportion of missing genotype data. Although we attempted to impute missing genotype information with the use of SAS, PHASE[70], MACH[55], and BEAGLE[57], these algorithms produced inconsistent results, and imputation on a simulated dataset with LD and missing data patterns modeled on the IWPC dataset produced unacceptably high error rates for some of the SNPs. We therefore abandoned this approach.

Genotypes for -4451C>A were missing in 82% of participants. Given the high rate of missing data for this SNP, and the fact that it showed little association with warfarin dose in univariate analysis, it was excluded from haplotype inference. Therefore, haplotype inference was restricted to participants with complete genotype data for the remaining 6 *VKORC1* SNPs (-

1639G>A, 497T>G, 1173C>T, 1542G>C, 2255C>T, 3730G>A). Complete genotype information on all 6 SNPs was available for 1168 chronic warfarin users, thus restricting the association between *VKORC1* haplotype and dose to 247 Asians, 365 blacks, and 556 whites. Haplotypes with frequencies less than 1% in all groups were combined into a single category. The informativeness of *VKORC1* haplotypes in a multivariable model was compared with that of the -1639G>A SNP alone in a similar multivariable model with the use of the F statistic. To allow valid comparison between the single SNP- and haplotype-based models, both analyses were conducted in the same set of subjects.

To assess the effect of *VKORC1* -1639G>A MAF on R^2 (proportion of variance in warfarin dose explained), genotypes with given MAFs were simulated for a cohort of 200 patients. The mean dose (and standard deviation) for each unique -1639G>A genotype from the entire IWPC cohort, irrespective of race, was used to randomly draw a normally distributed dose given each patient's individually simulated genotype. This was repeated 1000 times per MAF, and for each simulated study R^2 was calculated with the use of linear regression. The resulting R^2 distribution of the 1000 simulations was summarized with descriptive statistics. This process was repeated for MAFs ranging from 0 to 1.

To generate a worldwide survey of *VKORC1* haplotype frequencies, *VKORC1* haplotypes were inferred from genotype data for 6 SNPs (-1639G>A, 497T>G, 1173C>T, 1542G>C, 2255C>T, 3730G>A) for a total of 8751 subjects.

Note that the nomenclature used for the SNPs included in this paper is different than in the other papers (the conversion to rs-numbers is shown in Table 5). In the results section, rs-numbers are used.

Table 5. *Rs-numbers for the SNPs investigated in Paper III*

Nomenclature used in Paper III	Rs-number
-1639G>A	rs9923231
1173C>T	rs9934438
1542G>C	rs8050894
2255C>T	rs2359612
-4451C>A	rs17880887
497T>G	rs2884737
3730G>A	rs7294

Paper IV

Quality control (QC) procedures were applied on the whole genome data. In short, SNPs were excluded if the call rate was less than 98% or MAF was less than 1%. The 3405 SNPs that deviated from HWE ($p < 10^{-6}$) were flagged, but not excluded from analyses. Patients were excluded if their total call rate was less than 98%, if self-reported sex did not match genetic sex or

if the genetic relatedness to another patient was high (pi_hat from PLINK > 0.2). Following the QC, 554,725 SNPs in 2,962 patients were available for analysis of which 956 patients were in the warfarin arm. All subsequent analyzes are reported on the 956 patients and 554,725 markers passing QC control from the warfarin arm. Reported genetic coordinates are based on the NCBI human genome build 36. Data management and quality control were performed using PLINK.[71]

To account for possible population stratification all genome-wide analyses were adjusted for the first four genetic principal components.[59] Clusters were identified using k-means clustering (Hartigan and Wong method with k=6) on the four first genetic principal components and genome-wide significant findings were reanalyzed in the largest cluster identified by this method for consistency in beta estimates.

Regions with genome-wide hits were imputed, one megabase upstream and one megabase downstream, to enrich with SNPs using publicly available reference panels. Impute v2.2.2 was used for the imputation[52, 56] and SHAPEIT was used for pre-phasing the RE-LY genetic data.[72] The 1000 Genomes Phase I integrated variant set (February 2012) was used as reference set for the imputations.[53]

Genotyping

Paper I

All SNPs but 2 were genotyped with Homogeneous Mass Extend and iPLEX assays (Sequenom, Hamburg, Germany). Genotyping of 2 functional *CYP2C9* polymorphisms was performed using the ABI PRISM 7500 Sequence Detection System and TaqMan Pre-Developed Assay Reagent kits for Allelic Discrimination (Applied Biosystems, CA). All genotyping was performed at the Wellcome Trust Sanger Institute, Hinxton, United Kingdom.

Paper II and Paper III

Genotyping was performed with different methods for the different studies of Paper II and III; however quality control of the genotyping was performed by regenotyping 480 samples (approximately 10% from each study). This was done using two different methods. *CYP2C9* *2 and *3 and *VKORC1* rs9923231 were genotyped with the use of the TaqMan allelic discrimination assay (Applied Biosystems), whereas the remaining *VKORC1* SNPs were genotyped by mass spectrometry with the use of MassARRAY (Sequenom). The National Genotyping Center at the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, performed the re-genotyping.

Paper IV

Patients were genotyped with the Immunina Human610-quad DNA analysis BeadChip, which has 620,901 markers, using the Illumina Infinium II assay.[73] Genome calling were made using the Illumina GenomeStudio 2009.2 and the Illumina reference clusters based on signal intensities of 280 HapMap samples. All genotyping was performed at the SNP&SEQ Technology Platform in Uppsala.

Ethical committee approval

Study I was approved by the Karolinska Institutet Ethics Committee and regional ethics committees concerned. The Uppsala study, used in Study I, II and III, was approved by the ethics committee of Uppsala University. Study IV was approved by the ethics committees concerned in all countries including patients for the RE-LY genomics substudy.

Results

Paper I

Background

This paper is an extension of the previously published pilot study on warfarin dose and the effects of candidate genes (Paper B). In the pilot study, 201 Swedish patients were genotyped for SNPs in 29 candidate genes. SNPs in the genes *CYP2C18*, *CYP2C19*, *PROC*, *APOE*, *EPHX1*, *CALU*, *GGCX*, and *ORM1* were associated ($P < 0.05$) with warfarin dose, and so were haplotypes of *CYP2C19*, *PROC*, *GGCX*, *ORM1-2*, *PROZ*, *F7*, *F9*, and *NR1I2*.

This study aimed to produce reliable results from 29 candidate genes by genotyping functional SNPs and tagSNPs in a large prospectively collected warfarin cohort. We also tested for association with response to warfarin during initiation of warfarin, and generated a dosing algorithm for warfarin using demographic, clinical and genetic factors.

Results

In total, 1523 patients were included in the WARG study. Genotyping results were obtained from 1496 patients aged 18 to 92 years. Most patients were monitored for at least 3 months. The median follow-up time was 6 months, and the aggregated follow-up time 1214 warfarin-exposed patient-years. The number of patients that reached stable anticoagulation at least once during the study period was 1,324 (88 %) whereas 850 patients (57 %) also achieved stable dose at least once. We chose to use the first definition as our primary dose outcome since the latter excluded too many patients. Further, patients were on 100 other drugs that potentially interact with warfarin. Of the 1496 patients, 52 % used no interacting drug, 3.7 % used a drug that decreases the effect of warfarin and 46 % used a drug that increases the effect of warfarin. Median TTR during the first three months was 70 % whereas the median TTR during the entire treatment period was 74 %.

Replication results for association with maintenance dose are shown in Table 6. SNPs in *VKORC1* and *CYP2C9* that passed the stringent threshold (M_{eff}) in the pilot study (Paper B) were also significant in the WARG study, but other SNPs, for example rs2069919 in the gene *PROC* that showed a promising result in the pilot study ($R^2 = 9.0$ %, $p = 2.4 \times 10^{-4}$) did not replicate in the WARG study ($R^2 = 0.2$ %, $p = 2.1 \times 10^{-1}$).

Table 6. *Replication results*

Genetic variant or other factor		Pilot cohort (Paper B)			Replication cohort (Paper I)		
		N	R^2	P	N	R^2	P
VKORC1	rs9923231	181	31.7	$1.91x10^{-15}$	1293	29.3	$1.03x10^{-97}$
VKORC1	rs2359612	200	29.0	$2.30x10^{-15}$	1292	29.8	$9.82x10^{-100}$
CYP2C9	*2 and *3	201	15.9	$2.29x10^{-6}$	1321	11.8	$6.63x10^{-34}$
CYP2C9	rs4917639	197	11.8	$4.94x10^{-6}$	1092	11.7	$4.61x10^{-30}$
PROC	rs2069919	182	9.0	$2.16x10^{-4}$	1294	0.2	$2.07x10^{-1}$
EPHX1	rs4653436	196	4.8	$8.48x10^{-3}$	1207	0.1	$4.25x10^{-1}$
GGCX[a]	rs12714145/						
	rs7568458	198	3.4	$3.32x10^{-2}$	1252	0.1	$4.97x10^{-1}$
ORM1	rs1687390	149	2.6	$4.96x10^{-2}$	1310	0.0	$9.14x10^{-1}$
Age		201	9.2	$1.20x10^{-5}$	1324	14.5	$8.00x10^{-47}$
Gender		201	2.6	$2.29x10^{-2}$	1324	1.2	$6.89x10^{-5}$
Interaction (Increased INR)		201	3.6	$6.67x10^{-2}$	1324	1.5	$8.00x10^{-6}$

A prediction model was developed incorporating the replicated SNPs in *VKORC1* (rs9923231) and *CYP2C9* (*2, *3), age, gender and a variable counting the number of drugs that inhibit warfarin metabolism (increase INR). The predicted weekly dose was calculated by the algorithm [9.46832 - (0.90112 for rs9923231 A/G) – (2.01863 for rs9923231 A/A) – (0.50836 for *1/*2) – (0.97546 for *1/*3) – (1.10204 for *2/*2) – (1.74761 for *2/*3) – (3.40061 for *3/*3) – (0.03686 x age) – (0.27698 for females) – (0.06992 x number of drugs that increase INR)]^2. Through cross validation the R^2 of the model was 58.1 % (95 % CI 52.4 % – 63.4 %) and when applying it on the Uppsala study the R^2 was 52.8 %.

VKORC1 rs9923231 and *CYP2C9* *3 were associated with over-anticoagulation (INR > 4) during the first 5 weeks of treatment. The hazard ratio for *CYP2C9* *3/*3 compared to *1/*1 was 21.84 (95 % CI 9.46 - 50.42) and for *VKORC1* the hazard ratio for A/A vs. G/G was 4.56 (95 % CI 2.85 - 7.30).

We were not able to show that any of the genes studied had an effect on time to stable anticoagulation, but both *VKORC1* and *CYP2C9* had effect on TTR in total and during the first three months. The difference in TTR between the *VKORC1* rs992321 genotypes A/A and G/G was 6 % for both time periods. For *CYP2C9* *2/*3, the genotype *3/*3 was associated with lower TTR measures for both time periods then the other genotypes. None of the results for TTR passed the significance threshold for multiple tests.

Bleeding during warfarin treatment occurred in 146 patients (9.8 %) and serious bleeding occurred in 28 (1.9 %) patients. One of eight patients with the *CYP2C9* *3/*3 genotype had a serious bleeding during the first 5 weeks (p = 0.066).

Paper II

Background

Algorithms for predicting appropriate dose of warfarin are usually based on relatively small clinical populations and their general predictive ability is uncertain. The aim of the first paper from the IWPC group was to estimate a prediction model and its performance in an ethnically diverse dataset.

Data was collected on clinical factors that have previously been associated with warfarin therapy and that were available from all or most sites. These data included information on demographic characteristics, the primary indication for warfarin treatment, the stable therapeutic dose of warfarin, the treatment INR (the INR achieved with a stable warfarin dose), the target INR (the desired INR), the use of concomitant medications (grouped according to those that increase and those that decrease the INR), and the presence of genotype variants of *CYP2C9* (*1, *2 and *3) and at least *VKORC1* rs9923231 or one of six SNPs in LD with rs9923231.[43]

Results

The analysis setup was a "statistical race"; the research groups that took part in the study could use their favorite statistical approach to estimate a model resulting in a variety of methods used.

My approach was to use linear regression and a "keep it as simple as possible" modeling strategy. First medications were grouped based on inducing/inhibiting the effect of warfarin based on prior knowledge (Similar to Paper I). Then the analysis was performed including the following steps for each ethnic group and for all groups together:

1. Drop variables from further analyses based on number of missing values
2. Estimate univariate effects on the square-root of dose
3. Variables with estimated $R^2 > 1$ % were included in the initial model and based on prior knowledge and evidence of effect, variables with $R^2 < 1$ % could be also be included
4. Run stepwise AIC to remove less important parameters (however, carefully inspecting every step)
5. Clean final model from variables with small parameter estimates and high standard errors.

This resulted in four models where the model derived on all data performed better in each ethnic group then the models derived separately for the ethnic groups thus the model derived on all data was selected as the final model.

When comparing the different modeling approaches, the models that performed the best were a support vector regression model and the linear re-

gression model developed by me. The linear regression model was chosen as the final model since it contained fewer variables than the support vector model and was easier to both interpret and explain to a clinical public. The model (Table 7) included age, height, weight, amiodarone, use of inducers (carbamazepine, phenytoin, rifampin or rifampicin), ethnicity, *VKORC1* (rs9923231) and *CYP2C9* (*2/*3).

Comparisons were made between the derived pharmacogenetic prediction model, a clinical model (also built with linear regression) and a fixed dose approach (5 mg of warfarin per day). The pharmacogenetic model performed better than the two other approaches. Results for the validation set were; pharmacogenetic model MAE 8.5 mg/week and R^2 43 %, clinical model MAE 9.9 mg/week and R^2 26 % and fixed dose MAE 13.0 mg/week and R^2 0 %. A calibration plot on the validation data is shown in *Figure 6*. The pharmacogenetic model increased the performance due to better prediction in the low and high dose groups (defined as less or equal to 21 mg/week or greater or equal to 49 mg/week).

The NNG to predict a dose within 20 % of the observed dose with the pharmacogenetic model compared to the clinical model was 13 patients. For the pharmacogenetic model compared to the fixed dose approach the NNG was 6.

Table 7. *Pharmacogenetic prediction model derived in paper II, the IWPC model*

Sign	Coefficient	Variable
	5.6044	
-	0.2614 x	Age in decades
+	0.0087 x	Height in cm
+	0.0128 x	Weight in kg
-	0.8677 x	*VKORC1* A/G
-	1.6974 x	*VKORC1* A/G
-	0.4854 x	*VKORC1* genotype unknown
-	0.5211 x	*CYP2C9* *1/*2
-	0.9357 x	*CYP2C9* *1/*3
-	1.0616 x	*CYP2C9* *2/*2
-	1.9206 x	*CYP2C9* *2/*3
-	2.3312 x	*CYP2C9* *3/*3
-	0.2188 x	*CYP2C9* genotype unknown
-	0.1092 x	Asian race
-	0.2760 x	Black or African American
-	0.1032 x	Missing or mixed race
+	1.1816 x	Enzyme inducer status[2]
-	0.5503 x	Amiodarone status
=	Square root of weekly warfarin dose	

[2] Enzyme inducer status = 1 if patient taking carbamazepine, phenytoin, rifampin, or rifampicin, otherwise zero

The performance of the model in different ethnic groups showed that whites had the highest R^2 values followed by Asians and black or African Americans (Table 8).

Table 8. *Performance of the pharmacogenetic model in three ethnic groups (Online supplementary material to Paper II, section S9b)*

	White	Asian	Black or African American
Derivation n	2233	1229	353
Derivation MAE	8.7 mg/week	6.4 mg/week	11.6 mg/week
Derivation R^2	44.9 %	32.9 %	25.9 %
Validation n	561	300	97
Validation MAE	8.7 mg/week	7.2 mg/week	12.0 mg/week
Validation R^2	39.5 %	34.2 %	19.2 %

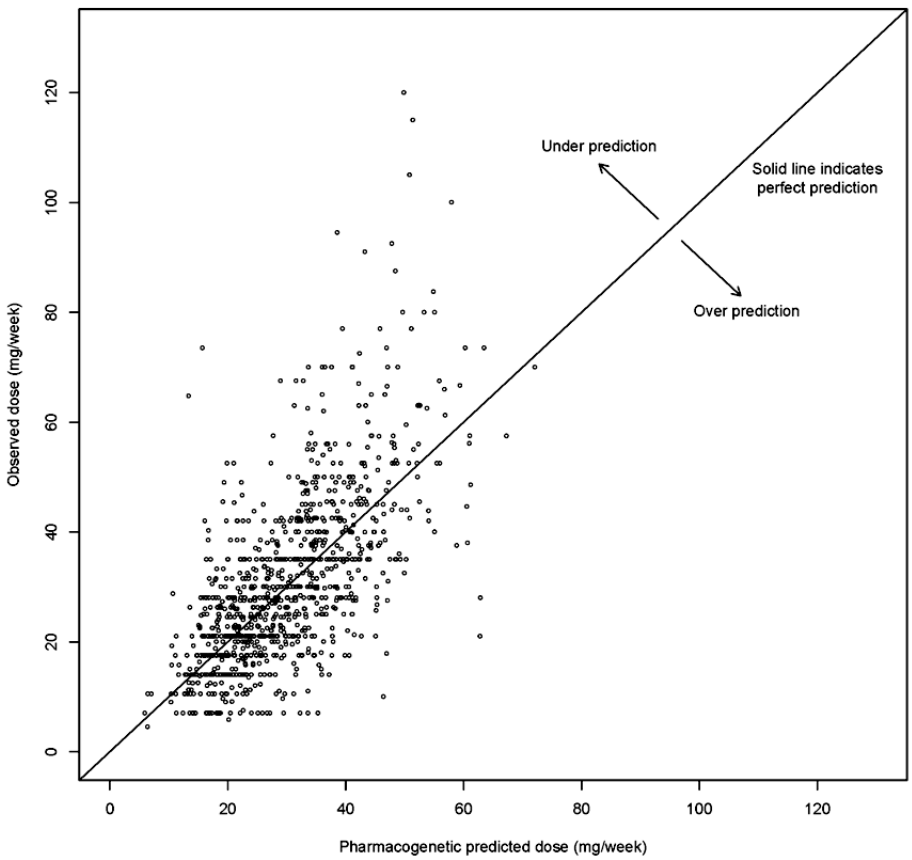

Figure 6. Calibration plot showing prediction results using the pharmacogenetic model on the validation data in paper II (Online supplementary material to Paper II, section S8). One patient with observed dose of 315 mg/week was excluded

Paper III

Background

Among whites and Asians, *VKORC1* polymorphisms have shown a consistently significant influence on warfarin response, accounting for 11 % to 32 % of the variability in dose (Paper A, Paper I).[33, 34, 74] Among North American blacks, *VKORC1* polymorphisms account for 4 % to 10 % of the variability in dose.[75, 76] Given that genetic diversity is known to be greater in persons of African descent,[77] investigators have hypothesized that other *VKORC1* polymorphisms, or combinations of multiple polymorphisms (haplotypes), may better explain the variation in dose in this group.

The second paper of the IWPC group investigated whether other *VKORC1* polymorphisms than rs9923231 (-1639G>A) could improve dose predictions in these ethnic groups.

Results

The IWPC dataset was expanded to include 6256 chronic warfarin users, incorporating an additional 20 Asians, 230 blacks, 302 whites, and 4 participants of unreported race. A total of 4886 patients of known race (1103 Asians, 670 blacks, and 3113 whites) with complete clinical data comprised the cohort for the current analyses. Indian subjects (n = 91) exhibited substantially different *VKORC1* allele frequencies compared with other Asians and were therefore excluded from all analysis.

There were significant differences in sex and age distribution, body size, and concomitant use of potentially interacting medications among racial groups. Warfarin dose requirements were significantly higher among blacks and lower among Asians compared with whites ($P < 0.001$).

Genotype distributions for *CYP2C9* and *VKORC1* were in HWE within each racial group. Although genotype frequencies differed significantly across racial groups ($P < 0.001$ for all SNPs), both *CYP2C9* and *VKORC1* showed significant association with dose in all racial groups.

As previously reported, LD between *VKORC1* SNPs was very strong among whites and Asians but weaker among blacks. However, 2 SNPs (rs9923231 and rs9934438) showed strong LD in all races ($r^2 = 0.97$ in Asians, $r^2 = 1.00$ in blacks, and $r^2 = 0.99$ in whites).

The performance of the IWPC model (derived in Paper II) was evaluated in each of the 3 racial groups in the expanded cohort. The variability in warfarin dose explained by the algorithm (as measured by the coefficient of determination, R^2) was higher in whites ($R^2 = 40.0$ %) than in Asians and blacks ($R^2 = 23.9$ % and $R^2 = 24.8$ %, respectively).

To determine which of the 7 *VKORC1* SNPs individually explains the highest proportion of variability in warfarin dose, the influence of each SNP on dose was assessed with the use of univariate analysis in all subjects with

genotype information for that SNP. Although providing a general indication of SNPs with large and small effects, the resulting R^2 values cannot be directly compared between SNPs, because different subsets of subjects were genotyped for each SNP as a result of different subsets of the 7 *VKORC1* SNPs having been genotyped at each contributing site. Therefore, to ensure valid comparisons of predictive ability of individual SNPs, the 4 SNPs (rs9923231, rs9934438, rs8050894, rs2359612) showing the strongest association with dose were further assessed in a subset of 1690 participants with genotype information for all 4 SNPs. Comparing R^2 between univariate models, multiple models and models including estimated haplotypes led to the result that rs9923231 (or rs9934438 which is in complete LD with rs9923231) performs the best for warfarin dose prediction across all investigated ethnicities (Table 9).

Table 9. MAF and variation in warfarin dose explained (R^2) by individual *VKORC1* SNPs among Asians, blacks and whites.

SNP	N	MAF %	Univariate R^2 %	†Multivariable R^2 %
rs9923231				
Asian	641	91.03	18.4	26.7
Black	368	10.05	4.2	26.3
White	681	37.81	22.5	48.5
rs9934438				
Asian	641	91.03	18.4	26.7
Black	368	10.19	4.5	26.2
White	681	37.81	22.8	48.9
rs8050894				
Asian	641	91.03	18.4	26.7
Black	368	26.36	0.2	23.0
White	681	38.33	22.4	48.3
rs2359612				
Asian	641	91.03	18.4	26.7
Black	368	21.33	2.6	24.7
White	681	37.89	22.5	48.4

N denotes number of participants with genotype information on all four *VKORC1* SNPs.
MAF denotes frequency of the minor allele.
Model parameters are estimated on the square root of warfarin dose and the resulting R^2 value is calculated by squaring the correlation between the retransformed predicted dose and observed dose.
†Multivariable R^2 analysis is restricted to 1638 participants (619 Asian, 354 blacks, and 665 whites) with genotype information on all four *VKORC1* SNPs and complete data on age, height, weight, *CYP2C9*, enzyme inducer status and amiodarone status.

We were also able to show that the variation in R^2 between ethnicities (22.5% in white, 18.4% in Asian and 4.2% in African American) was due to differences in the MAF of the investigated polymorphism rs9923231 (38.8% in white, 91.2% in Asian and 10.8% in African American for the minor allele A). This was shown by simulating the effect of MAF on R^2 (*Figure 7*).

Given the importance of MAF in determining the effect of *VKORC1* on warfarin dose at a population level and recognizing global population diversity, we undertook a worldwide survey of *VKORC1* haplotype frequencies. Haplotypes from 8751 subjects (including 1168 with warfarin data) representing global population diversity were summarized by the subjects' country of origin. Haplotype distribution within the Asian, black, and white racial groups was consistent across geographically separated populations and differed substantially among the 3 racial groups. Compared with other Asian groups, South Indians showed substantial differences in haplotype distribution, resulting in their exclusion from the analysis of the effect of *VKORC1* on warfarin dose.

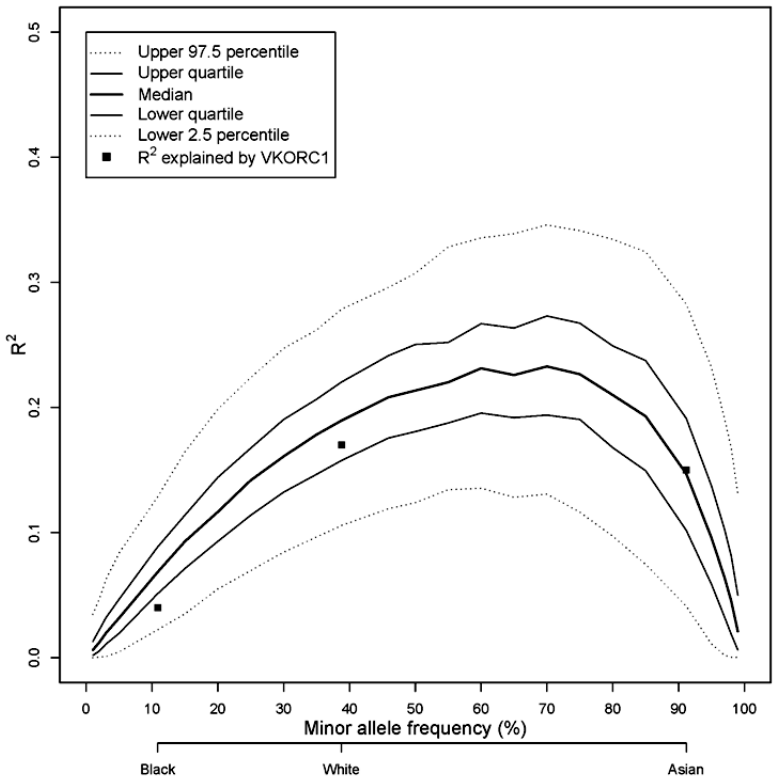

*Figure 7.*Effect of *VKORC1* rs9923231 MAF in explaining warfarin dose variability at the population level. Results of a simulation study showing the influence of the frequency of the A allele for rs9923231 on the amount of variance in warfarin dose explained (R^2). Quartiles and upper and lower 2.5th percentiles of the distribution of R^2 values from the simulation are plotted as a function of minor allele frequency. The actual R^2 estimates for each racial group in the IWPC data are plotted as squares at the observed MAF for each group.

Paper IV

Background

Risk of major bleeding during warfarin treatment is related to the intensity of anticoagulation although most bleedings on warfarin occur while within therapeutic target range with INR between 2.0 and 3.0.[1] The major risk factors for bleedings on warfarin are age, renal function, concomitant antithrombotic medication, concomitant diseases and unstable and high INR level (above 3.5) e.g. because of genetic variation.[1, 18] The maintenance dose model derived by the IWPC (Paper II) explained 43% of the variability in dose. Although the IWPC model performs well, factors shown to affect warfarin dosing like renal function, smoking and *CYP4F2* could not be evaluated since they were either not available or had too much missing data.[31, 78]

We hypothesized that there could be genetic risk factors associated with the efficacy and safety of warfarin treatment, and that genetic markers other than *CYP2C9*, *VKORC1* and *CYP4F2*, might affect warfarin maintenance dose. We analyzed the genome in relation to the primary efficacy and safety endpoints of the RE-LY study, TTR and maintenance dose. We also investigated the clinical effects of the previously known genes *VKORC1* and *CYP2C9* on TTR and risk of major bleeding. Further, we investigated the performance of the IWPC prediction model and if any additional variables (renal function, smoking, *CYP4F2*) might improve performance of future prediction models.

Results

The number of warfarin treated patients available for analysis after QC was 956. These patients had a median follow-up of 816 days. Eighty one % had a history of VKA use and 40 % were warfarin naïve at baseline (duration of previous VKA treatment ≤ 2 months). The maintenance dose of warfarin could be calculated in 951 patients and had a median value of 29.6 mg/week. Overall median TTR was 72.2% (mean 70.1%) whereas the median TTR in the first three months was 61.7% (mean 61.0%). In the 956 patients there were 24 stroke or systemic embolism events and 53 major bleeding events reported corresponding to an incidence rate of 1.2% and 2.6% respectively per patient year.

Genome-wide results

Genome-wide significant hits were found for stroke or systemic embolism and for TTR during the whole treatment period (Table 10). Concerning warfarin maintenance dose the regions around *CYP2C9* (on chromosome 10) and *VKORC1* (on chromosome 16) gave strong signals when adjusting for clinical factors (*Figure 8* A). When, in addition to the clinical factors, also adjusting for *CYP2C9*/*VKORC1* we found two novel findings*;* one rare SNP

in the gene *DDHD1* on chromosome 14 and one more common variant in the gene *NEDD4* on chromosome 15 (*Figure 8* B).

Table 10. *Genome-wide significant findings in Paper IV*

Outcome	SNP	Chr	Gene	MAF	Beta (95 % CI)	P
Warfarin dose	rs10871454	16	*STX4*	37.9	-0.82 (-0.91;-0.72)	4.40×10^{-64}
Warfarin dose	rs9923231	16	*VKORC1*	38.0	-0.82 (-0.91;-0.72)	7.26×10^{-64}
Warfarin dose	rs4917639	10	*CYP2C9*	19.4	-0.72 (-0.84;-0.60)	8.47×10^{-32}
Warfarin dose	rs1057910 (*3)	10	*CYP2C9*	6.6	-0.93 (-1.11;-0.74)	1.77×10^{-22}
Warfarin dose	rs1799853 (*2)	10	*CYP2C9*	12.4	-0.48 (-0.62;-0.34)	4.98×10^{-11}
Warfarin dose (adjusted)	rs2288344	15	*NEDD4*	30.2	0.20 (0.13;0.28)	3.96×10^{-8}
Warfarin dose (adjusted)	rs17126068	14	*DDHD1*	1.4	-0.80 (-1.09;-0.52)	4.14×10^{-8}
TTR in total	rs4379440	8	*ASPH*	7.6	-6.82 (-9.27;-4.37)	5.00×10^{-8}
TTR in total	rs17791091†	8	*ASPH*	6.9	-7.84 (-10.45;-5.23)	4.13×10^{-9}
Stroke or systemic embolism	rs6509967	19	*NLRP4*	1.0	3.15 (2.13;4.17)	1.47×10^{-9}

† Imputed SNP

IWPC prediction model performance

Applying the IWPC prediction model for warfarin maintenance dose (Paper II) gave a R^2 of 50.9 % and a MAE of 6.59 mg / week. Of the 951 patients with dose information available, 534 patients (56.2 %) were predicted the ideal dose corresponding to prediction within 20 % of the observed maintenance dose. The number of patients with an observed maintenance dose ≤ 21 mg/week, > 21 mg/week to < 49 mg/week and ≥49 mg/week were 215, 648 and 88, respectively; the percentage of ideal dose predictions within those categories were 46.0 %, 63.9 % and 23.9 %, respectively.

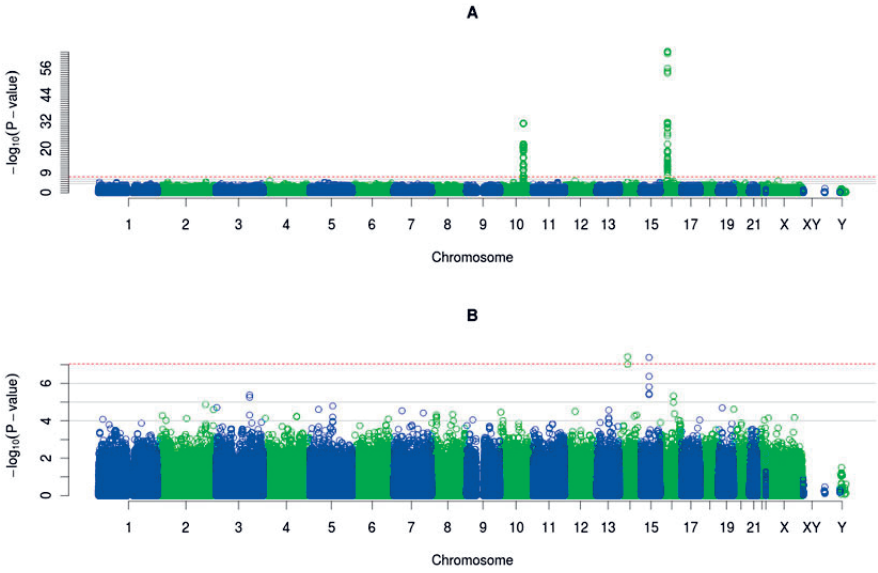

Figure 8. **(A)** Manhattan plot for the analysis of warfarin maintenance dose adjusted for clinical factors and the first four genetic principal components. **(B)** Manhattan plot for the analysis of warfarin maintenance dose adjusted for clinical factors, genetic factors (*VKORC1* rs9923231 and *CYP2C9* *2/*3) and the first four genetic principal components. In (A) and (B) the red dashed line indicates the threshold for genome-wide significance

Evaluation of additional variables for dose prediction models

We evaluated the potential benefit of including any of the variables CrCl, smoking or *CYP4F2* rs2108622 as well as the novel SNPs found in the current GWAS. CrCl showed a large univariate effect with an R^2 of 9.6 % ($P = 6.30 \times 10^{-23}$), however, much of this effect was due to the correlation of CrCl with age (r = -0.60) and weight (r = 0.69). When adding CrCl to a base model including the variables used in the IWPC model (Paper II), the added R^2 was 0.4 % but CrCl was still significant (P = 6.51 x 10-4). As an example the lowering of dose for patients moving from the median CrCl in this study of 73 mL/min to 30 mL/min is -1.3 mg/week (-13.1 %) for patients having a 10 mg/week dose, -2.3 mg/week (-7.7 %) for patients having a 30 mg/week dose, and -3.0 mg/week (-6.0 %) for patients having a 50 mg/week dose. However, note that CrCl < 30 was an exclusion criterion in the RE-LY study which could impact the results. Smoking showed no signs of being important for determination of warfarin dose. Also, the beta estimate of smoking is negative (-0.102) which is contradictionary to a study by Gage et al., which estimated a 10% increase in dose for smokers.[33] All three SNPs evaluated (*CYP4F2* rs2108622, *DDHD1* rs17126068 and *NEDD4* rs2288344) had statistically significant effects and showed a univariate R^2 of ≈ 1% and added roughly the same amount to the base model.

The clinical value of adding the variables to a base model including the variables used in the IWPC model was estimated. We found increased predictive values of *CYP4F2*, *DDHD1* and *NEDD4* when added to a model with clinical factors and *CYP2C9*/*VKORC1*. The NNT to prevent one patient from being predicted worse than 20% of the actual dose was 106 when adding *CYP4F2*, *DDHD1* and *NEDD4*. For comparison, adding *CYP2C9* *2/*3 and *VKORC1* rs9923231 to a clinical model including all non-genetic variables from the IWPC model gave a NNT of 7, and an illustration of the performance of this model compared to the clinical model is shown in *Figure 9*.

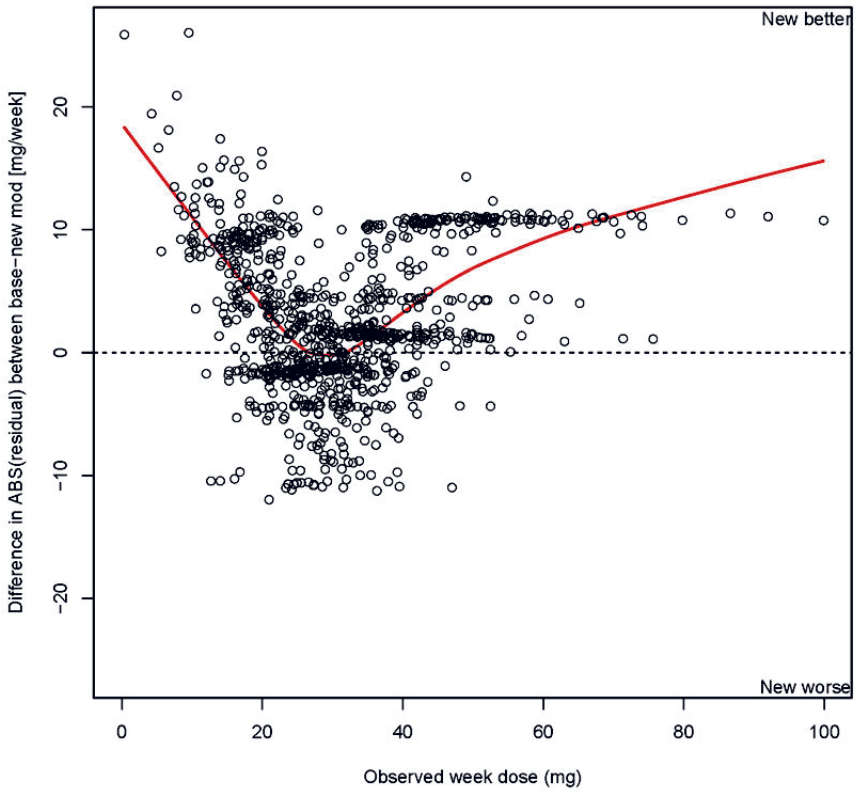

Figure 9. Added value of *CYP2C9* *2/*3 and *VKORC1* rs9923231 to a base prediction model including the clinical variables from the IWPC algorithm; age, height, weight, amiodarone, inducer use and ethnicity. Each dot illustrates a patient's difference in absolute residual between the base model and the new model. The red line is a loess smother showing the trend in the data

Paper V

Background

The aim of Paper V was to provide a broad review of the field of warfarin pharmacogenomics with special focus on warfarin dose prediction models and how they could be used.

Results

There are two key time periods when treating a patient with warfarin and both have available pharmacogenetic prediction models. The periods are initiation, which is when you give starting doses to the patient and dose revision, which is when you adjust the dose given the INR response.

The initiation model by Avery et al. discussed in Paper V uses a predicted maintenance dose as input and combines this dose with estimated elimination rate constants of the *CYP2C9* *2/*3 genotypes (Table 11).[79] The predicted maintenance dose, used as input (*MD*) could for example be calculated using the IWPC model (Paper II). Example loading doses for different genotypes of *CYP2C9* and *VKORC1* are shown in Table 13.

Table 11. *Loading dose estimation according to Avery et al.*[79]

Model type: Loading dose estimation

$$x = MD * \left[\frac{\frac{1}{\left(1 - e^{-k*\tau}\right)} - \left(1 + e^{-k*\tau} + e^{-k*2\tau}\right)}{\frac{1}{3} + \frac{2 * e^{-k*\tau}}{3} + e^{-k*2\tau}} \right]$$

Where MD is the calculated maintenance dose per day in mg. Could be calculated by the IWPC model (Paper II) (note that the IWPC model gives weekly dose, divide by 7 to get the day dose).

k is the elimination rate constant for the *CYP2C9* genotypes: *1/*1 = 0.0189 h^{-1}, *1/*2 = 0.0158 h^{-1}, *1/*3 = 0.0132 h^{-1}, *2/*2 = 0.0130 h^{-1}, *2/*3 = 0.009 h^{-1}, *3/*3 = 0.0075 h^{-1}

τ is the warfarin dosing interval, use 24 (24 h).

The initial doses on day 1-3 are then calculated as: Day 1 = MD + x, Day 2 = MD + 2x/3, Day 3 = MD + x/3

As an example of a dose refinement model, the model developed by the International Warfarin Dose Refinement Collaboration (IWDRC) was used.[80] The model, shown in Table 12, uses information on previous doses and the INR value on day four or five to estimate the revised maintenance dose. The same group has recently published a model for dose revision after 6-11 days; however this model was in press during the preparation of the manuscript and was not included.[81] The added clinical value of this model

could for instance be if a patient only is available for INR testing later than day 4-5 of therapy.

Table 12. *Dose refinement model according to IWDRC; Lenzini et al.*[80]

Model type: Dose refinement
Dose (mg/week) = EXP (3.10894 − 0.00767 × age − 0.51611 × ln(INR) − 0.23032 × rs9923231 − 0.14745 × *CYP2C9**2 − 0.3077 × *CYP2C9**3 + 0.24597 × BSA + 0.26729 × Target INR −0.09644 × African origin − 0.2059 × stroke − 0.11216 × diabetes − 0.1035 × amiodarone use − 0.19275 × fluvastatin use + 0.0169 × $dose_{-2}$ + 0.02018 × $dose_{-3}$ + 0.01065 × $dose_{-4}$).
The result is on logarithmic scale and should be retransformed by the exponential function ($e^{predicted\ dose}$).
INR is the INR on day 4 or 5.
VKORC1 rs9923231 should be entered as 0 for homozygous G/G, 1 for heterozygous and 2 for homozygous A/A. *CYP2C9**2 and *CYP2C9**3 should be entered as 0 if absent, 1 if heterozygous and 2 if homozygous.
$Dose_{-i}$ is dose given i days before INR is measured.
Body surface area (BSA) is calculated according to the formula by Dubois & Dubois where BSA = $(weight\ (kg)^{0.425} \times height\ (cm)^{0.725})/139.2$
For African origin, stroke, diabetes, amiodarone use and fluvastatin use enter 1 if present otherwise 0

How these prediction models could be used in a clinical setting was shown with an example: a patient that is to be treated with warfarin due to AF and additional risk factors for stroke with a target INR of 2.5. The patient has the following characteristics; Caucasian, age of 60, height 175, weight 75, no interacting drugs and the genotypes *CYP2C9* *3/*3 and *VKORC1* A/G. To predict the initial doses for this patient first use a pharmacogenetic maintenance dose model, such as the IWPC model. Then use the formulas by Avery et al. to calculate the initial doses (Table 11 and Table 13). This patient would be predicted a maintenance dose of 11 mg/week (1.6 mg/day) and initial doses of 5.1mg, 3.9mg and 2.7mg on days 1-3 respectively. On day four the patient comes back for an INR test and has an INR of 2.0. At this point a dose revision model could be used, such as the model by Lenzini et al. (Table 12) which would predict a dose of 15.6 mg/week (2.2 mg/day).

Table 13. *Examples of pharmacogenetic loading doses using the formula by Avery et al and the IWPC pharmacogenetic prediction model.*[79]

				Loading doses‡		
CYP2C9	***VKORC1 rs9923231***	**IWPC† dose/week (mg)**	**IWPC dose/day (mg)**	**Day one (mg)**	**Day two (mg)**	**Day three (mg)**
*1/*1	A/A	24	3.43	5.51	4.82	4.12
*3/*3	A/A	7	1.00	3.23	2.49	1.74
*1/*1	A/G	33	4.71	7.56	6.61	5.66
*3/*3	A/G	11	1.57	5.07	3.90	2.74
*1/*1	G/G	44	6.29	10.1	8.83	7.56
*3/*3	G/G	18	2.57	8.3	6.39	4.48

† Approximate predictions are from the example nomogram in Figure 1 of Paper V for a Caucasian patient with no interacting drugs, age 60 years, height 175cm and weight 75 kg.
‡ Prediction of loading doses were made using k (elimination rate constant) of 0.0189 for *1/*1 and 0.0075 for *3/*3 as in Avery et al (Table 11).[79]

Discussion

We have shown that the required dose of warfarin to reach therapeutic INR levels (i.e. 2.0 - 3.0) is strongly related to SNPs in *VKORC1* and *CYP2C9*. The genetic markers together with clinical factors explain a large amount of the variability in dose. The amount of variability explained, in terms of R^2, varies depending on the mix of ethnicities in the data. In our studies the validated R^2 values of the IWPC model were in the range 43 % to 51 %. The greatest improvements in dose predictions with pharmacogenetic prediction models compared to clinical models can be seen in the low and high dose ranges (< 21 mg/week and > 49 mg/week) which indeed means that pharmacogenetic warfarin dosing is about tailoring the dose for the outliers.

Why predict warfarin dose

The ultimate goal of pharmacogenetics is to individualize therapy and warfarin is a good example of where pharmacogenetics could help tailor the dose from the beginning of treatment. The narrow therapeutic index of warfarin and high interindividual variation in dose needed is a challenge in clinical care (*Figure 10*).

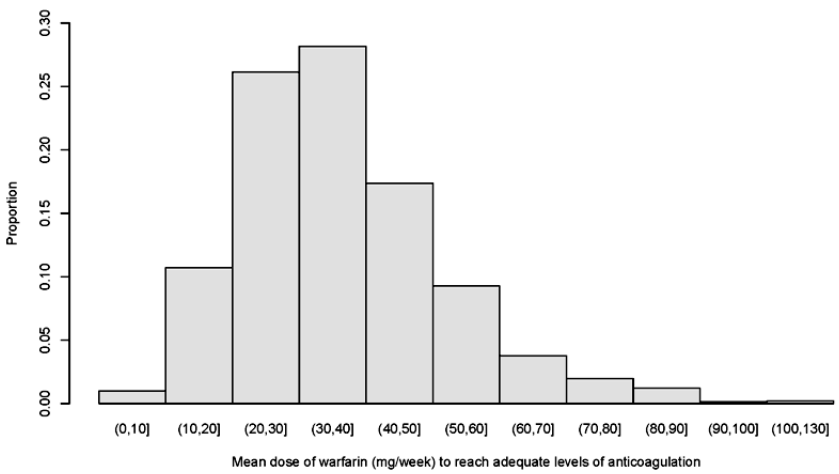

Figure 10. Distribution of mean doses needed (mg/week) to reach INR levels of 2.0 – 3.0. (Result from the WARG study, unpublished)

Complications from inappropriate warfarin dosing remain as one of the most common reasons for emergency room visits in the USA.[82, 83] Unintentional overdosing of warfarin is the most common cause of hospitalization due to adverse reactions in the USA, and the second most common cause in the UK.[84, 85]

There is evidence of a pronounced risk of bleeding during the initiation phase (1–3 months), which a genotype-guided dose has the potential to reduce.[1, 86] In Paper I, we analyzed the time to first INR > 4.0, which can be seen as a surrogate marker for early bleeding risk. Both *CYP2C9* *3 and *VKORC1* rs9923231 were associated with the outcome. The hazard ratio for *CYP2C9* *3/*3 compared to *1/*1 was 21.84 (95 % CI 9.46 - 50.42) and for *VKORC1* rs9923231 the hazard ratio for A/A vs. G/G was 4.56 (95 % CI 2.85 - 7.30).

Major genetic determinants of warfarin dose

The replication results in Paper I showed that the major genes affecting variability in warfarin maintenance dose are *CYP2C9* and *VKORC1*. None of the interesting results in Paper B, except for *CYP2C9* and *VKORC1*, replicated. In Paper IV we took the step, in a GWAS, from 183 SNPs in 29 candidate genes to 554,725 SNPs across the whole genome. Again the results showed the major impact of the polymorphisms in the *CYP2C9* and *VKORC1* regions (*Figure 8* A on page 54). This has also been seen in two previously published GWAS on warfarin maintenance dose, one by Takeuchi et al. on the WARG data (325,997 SNPs on 1,053 patients) and the other by Cooper et al. (538,929 SNPs on 124 patients).[87, 88]

Pharmacogenetic prediction models for warfarin dosing

There are three different types of prediction models for warfarin dosing. The first is the prediction of stable warfarin maintenance dose, which is the most studied outcome in warfarin pharmacogenetics. However, maintenance dose prediction models do not tell us how to initiate treatment, which leads us to the second type of model: prediction of starting doses. The third type of prediction model is the dose refinement model that also includes INR response after the initial doses.

The first prediction models for stable warfarin dose were estimated on relatively small populations mainly consisting of Caucasian patients included from one area or clinic.[34] In Paper I, a prediction model for warfarin dosing was derived on a large patient material, however there data mainly included Swedish patients and weight and height was not measured. This model was externally validated on Swedish patients and gave a R^2 of 53 %.

While these models might predict the dose within the same catchment area, they are probably not portable to a more broad population from other regions or countries.[89] To address the problem of generalizability, the IWPC

was established with the primary aim to develop an algorithm for warfarin dose prediction.

The IWPC model

The highly replicated genetic variants from *CYP2C9* and *VKORC1* were included in the IWPC data (Paper A, Paper I).[20, 33, 34, 42, 43] In Paper II a prediction model was developed on this ethnically diverse dataset including studies from 21 research groups around the world. The aim in the development of the model, called the IWPC model, was to keep the model as simple as possible. The main reason to keep the model simple was chosen because a simple model is both easier to interpret and to implement in a clinical setting. Although models were first developed for all patients and separately by ethnicity, the model developed on all performed better in each ethnic group then the specialized model for each group. However, the model performed best in whites compared to other ethnic groups.

The IWPC model (Table 7 on page 47) includes age, height, weight, amiodarone, use of inducers (carbamazepine, phenytoin, rifampin or rifampicin), ethnicity, *VKORC1* (rs9923231) and *CYP2C9* (*2/*3). The R^2 and MAE on the validation data in Paper II was 43 % and 8.5 mg/week, respectively. Several studies have externally validated the IWPC model together with other models, and the overall conclusion is that the IWPC model performs well; however, the sample size has been small in these validation studies.[90-92] In Paper IV the IWPC model performance was estimated in 951 patients and the results were R^2 and MAE of 51 % and 6.6 mg/week, respectively.

Drawbacks of current pharmacogenetic dosing algorithms, like the IWPC model, are that they underestimate patients with large dose requirements and overestimate a proportion of patients in the normal dose range (*Figure 6* on page 48).[33, 91] The cause of warfarin resistance, resulting in high warfarin doses, could for instance be a polymorphism, diet or nonadherence to take the medicine as prescribed. Studies have identified variants in *VKORC1* associated with warfarin resistance.[93] However, these rare mutations are probably not worthwhile to incorporate into prediction models for warfarin dosing because it would take hundreds of patients to improve the dosing of one patient. Furthermore, under prediction is generally not a problem in terms of safety when initiating patients on warfarin.

Ethnic differences in prediction results

The difference in performance of the IWPC model between ethnicities was evaluated in Paper III. Here the IWPC prediction model gave an R^2 of 24 % in Asians, 25 % in blacks and 40 % in whites. Much of the difference in model performance was shown to be due to differences in the MAF of the *VKORC1* rs9923231 SNP between ethnicities. While there is most variability in rs9923231 among whites (MAF = 38 % for the minor allele A), there

is a considerably lower allele frequency among blacks (MAF = 11 % for the minor allele A) and a shift between the minor allele and wildtype allele in Asians (MAF = 9 % for the minor allele G). This is also an explanation to the higher maintenance dose requirement among blacks (more G alleles = higher doses) and lower maintenance dose requirement among Asians (more A alleles = lower doses) compared to whites.

Despite the differences in amount of variability explained by *VKORC1* rs992321 between ethnicities, the prediction accuracy on a patient level is the same (i.e. similar decrease in dose per minor allele). However, on a population level, genotype driven dosing might not be as cost-efficient in countries where there is low variation in *VKORC1*. Although we did not have sufficient amount of warfarin data on Hispanics in the IWPC cohort to analyze them as a separate group, our data showing the consistency of genotype effects on dose across racial groups suggest that dose reductions associated with the *VKORC1* genotypes are likely to hold true in this ethnic group as well. Moreover, given our results showing the importance of the MAF and the high frequency of the rs9923231 A allele in this group (45.3%), the influence of *VKORC1* on warfarin dose refinement among Mexican Americans is likely to be substantial.

Additional genetic factors affecting dose other than CYP2C9 and VKORC1

In a GWAS we investigated if any additional genes affect warfarin dose other than *CYP2C9* and *VKORC1* (Paper IV). When adjusting for clinical factors together with *CYP2C9* and *VKORC1* we found two novel genes, *DDHD1* and *NEDD4*, affecting warfarin dose.

The *NEDD4* SNP rs2288344 was common with a MAF of 30.2 % and the adjusted beta estimate was 0.20 (increase in dose mg/week per minor allele, on square root scale). As an example the increase in dose for homozygote patients for the minor allele compared to homozygotes for the wild type allele was 2.7 mg/week (26.9 %) for patients having a 10 mg/week dose, 4.5 mg/week (15.1 %) for patients having a 30 mg/week dose and 5.8 mg/week (11.6%) for patients having a 50 mg/week dose.

The *DDHD1* SNP rs17126068 was rare with a MAF of 1.4 % and the adjusted beta estimate was -0.80 (lowering in dose mg/week per minor allele, on square root scale). Because of the low MAF there were no homozygotes for the minor allele. As an example the lowering in dose for heterozygote patients compared to homozygotes for the wild type allele was -4.4 mg/week (-44.2 %) for patients having a 10 mg/week dose, -8.1 mg/week (-27.1 %) for patients having a 30 mg/week dose and -10.7 mg/week (-21.3%) for patients having a 50 mg/week dose.

When comparing with a pharmacogenetic model including the variables in the IWPC model, the NNT to prevent one patient from being predicted worse than 20 % of actual dose for *NEDD4* was 136.

The effect of CYP4F2 on warfarin dose

The *CYP4F2* SNP rs2108622 that previously been shown to affect warfarin dosing was investigated in Paper IV.[78, 88, 94] The effect of rs2108622 was statistically significant and the adjusted beta estimate was 0.178 (increase in dose mg/week per minor allele, on square root scale). For *CYP4F2* the NNT to prevent one patient from being predicted worse than 20 % of actual dose was 317.

Combination of the novel SNPs found with CYP4F2

Combining the genetic variables from *DDHD1*, *NEDD4* and *CYP4F2* resulted in 57.9% of the patients were being predicted an ideal dose (within 20 % of observed dose). The NNT was 106 compared to a pharmacogenetic model including the variables in the IWPC model, i.e. genotyping these markers and adding them to the IWPC prediction model for warfarin dosing could prevent one in 106 patients from being predicted worse than 20 % of actual dose. In comparison, the NNT for adding *VKORC1* rs9923231 and *CYP2C9* *2/*3 to a clinical model was estimated as 7. Since no validation cohort is used these estimates are probably positively biased, hence the true NNT is most likely higher than the estimated NNT in this study. For example, the estimate of NNT in the IWPC publication, when comparing a clinical model to a pharmacogenetic, was 13 (Paper II). Given that adding the three genetic factors from *CYP4F2*, *DDHD1* and *NEDD4* gives a NNT above 100 while doubling the number of SNPs that has to be genotyped, one could hypothesize that it is probably not cost-effective in clinical practice. However, the cost-effectiveness relies on the genotyping costs.[95]

Additional clinical variables

Smoking had too much missing data to be included in the IWPC prediction model derived in Paper II, also, measures of kidney function (CrCl) were not available. Smoking has been associated with increased doses of 10% and CrCl is associated with 9.5 % lower doses in patients with moderate kidney impairment compared to no/mild impairment (CrCl 30-59 mL/min and CrCl $\geq$ 60 mL/min).[31-33] Both these variables were available in the RE-LY data used in Paper IV. Smoking had no statistically significant effect (beta -0.102 with 95 % CI -0.288 – 0.083). Notably, the estimated effect was opposite to the previous study. CrCl had a statistically significant effect with beta estimate of 0.005 per increase of one CrCl unit. As an example the lowering of dose for patients moving from the median CrCl in Paper IV of 73 mL/min to 30 mL/min was -1.3 mg/week (-13.1 %) for patients having a 10 mg/week dose, -2.3 mg/week (-7.7 %) for patients having a 30 mg/week dose and -3.0 mg/week (-6.0%) for patients having a 50 mg/week dose.

Dose predictions in children and young adults

As the prediction models derived in Paper I and II are estimated on adult patients, they are probably not suitable for predictions in children. Presently, many research groups are evaluating genetic effects on warfarin dose in children and a recent paper by Biss T.T et al. showed the current IWPC model constantly over estimates the warfarin dose in children by an average of 1.5mg/day.[96] They however showed that a similar proportion of the variation in dose was explained by *VKORC1* and *CYP2C9* in children as in adult patients.

Genetic effects on efficacy and safety

In Paper I and IV the genetic effects on major bleeding were investigated for *CYP2C9* *2/*3 and *VKORC1* rs9923231 and in addition a GWAS on stroke or systemic embolism and major bleeding was performed in Paper IV.

Tendencies of increased risk of early major bleeding (< 5 weeks from start of therapy) were seen for *CYP2C9* *2/*3 in Paper I but no increased risks were seen in Paper IV.

The GWAS in Paper IV on stroke or systemic embolism revealed a genome-wide significant signal in the *NLRP4* gene on chromosome 19 (rs6509967, adjusted hazard-ratio 19.1 per minor allele). The gene *NLRP4* (NLR family, pyrin domain containing 4) is thought to play a role in inflammation which might have relevance to cardiovascular diseases.[97] For major bleeding, there were no genome-wide significant signals.

Genetic effects on TTR

Both *CYP2C9* *2 and *3 and *VKORC1* rs9923231 were shown to affect TTR measures in Papers I and IV. Furthermore, a GWAS of TTR was conducted in Paper IV.

In Paper I, TTR in range 2.0 – 3.0 was analyzed for both *CYP2C9* *2/*3 and *VKORC1* rs9923231. During the first 3 months, the median TTR was 70 % (average, 67 %). *VKORC1* rs9923231 had effect on the outcome (P = 0.004), average TTR was the highest (71 %) in A/A individuals and the lowest (65 %) in G/G individuals. *CYP2C9* *2/*3 also affected this outcome (P = 0.001) where TTR was the lowest in *3/*3 individuals (average, 50 %). The median TTR during the entire treatment period was 74 % (average, 71 %). *VKORC1* rs9923231 was significantly associated with overall TTR (P = 0.001). As above, average TTR was the highest (75 %) in A/A individuals and the lowest (69 %) in rs9923231 G/G individuals. *CYP2C9* *2/*3 was associated with overall TTR (P = 0.002) where TTR again was lowest in *3/*3 individuals (58 %).

In Paper IV, TTR in range 2.0 – 3.0, TTR below 2.0 and TTR above 3.0 were analyzed for both *CYP2C9* *2/*3 and *VKORC1* rs9923231. The median TTR in the first three months was 61.7 % (mean 61.0 %) and the median

TTR during the whole treatment period was 72.2 % (mean 70.1 %). *VKORC1* rs9923231 was associated with both TTR below 2.0 and above TTR 3.0 during the first three months. Further, *CYP2C9* *3 was associated with TTR above 3.0 during the first three months. No other statistically significant associations were found.

Paper IV also reported the first GWAS on TTR. Concerning TTR during the whole treatment period, a genome wide hit was found for the SNP rs4379440 in the gene *ASPH* (aspartate beta-hydroxylase). The effect of rs4379440 on TTR was -6.8 % per minor allele. An imputed SNP in the same gene as the typed rs4379440, namely rs17791090, showed an even stronger effect on total TTR with -7.8 % per minor allele. The *ASPH* gene is thought to play an important role in calcium homeostasis. The gene is expressed from two promoters and undergoes extensive alternative splicing. The longest isoforms (a and f) include a C-terminal domain that hydroxylates aspartic acid or asparagine residues of some proteins, including protein C, FVII, FIX, and FX, and the complement factors C1R and C1S.[97]

Clinical implications

Warfarin treatment in Sweden

The quality of warfarin treatment in Sweden is world leading. In RE-LY, the mean TTR of patients included in Sweden was 77 % and in a study on 18,391 patients from AuriculA, the Swedish national registry for AF and anticoagulation, the reported TTR was 76 %.[98, 99] Given the generally high quality of warfarin treatment in Sweden, the question is whether personalized anticoagulant therapy could further increase the safety and efficacy of anticoagulant therapy. The results from Paper I are based on Swedish patients. In this study the TTR within three months of treatment start was 67 % and the overall TTR was 71 %. Despite the high treatment quality, there were still genetic effects of *CYP2C9* and *VKORC1* on time to first INR > 4.0 and TTR, as mentioned earlier.

Although pharmacogenetic dosing has the potential to further increase the overall quality of Swedish warfarin treatment, patients with concomitant diseases or drugs might benefit the most from the personalized therapy approach, and could be selected for genotyping. When choosing between new OAC and warfarin, selection of treatment drug could be made based on genotype, measures of CrCl, and estimates of bleeding risk such as the HAS-BLED score.[26] Patients extremely sensitive to warfarin could be recommended new oral anticoagulants (NOAC), while patients not eligible for treatment with NOAC, based on treatment recommendations, could be treated with genotype driven warfarin treatment.

Limitations

Despite the strengths of the studies presented, there are also limitations.

Study I did not include measures of height and weight and none of the studies presented have included estimates of vitamin-K intake through diet. If vitamin-K intake could be estimated with high precision, it could be of value. A study by Pavani et al. included vitamin-K intake in a pharmacogenetic prediction model for warfarin dosing.[100] Here the information on vitamin-K intake was estimated by collecting information on vegetables and other foods that contain vitamin-K. However, in their multiple model the parameter estimate for vitamin-K intake was not statistically significant ($p = 0.35$), which could be caused by a combination of low power ($n=125$) and/or low precision in the estimated vitamin-K intake.

In Study I and II measures of CrCl was not available which has been shown to affect warfarin dosing.[31, 32]

Study II and III had problems with nonrandom missing value patterns of phenotypes and genotypes caused by different sites not providing information on some of the variables. For example, information on smoking was missing in 27 % of whites, 83 % of Asians and 5 % of black or African American patients. In Study III missing genotypes of the *VKORC1* SNPs forced us to investigate the performance in subjects with complete information on some of the SNPs which lowered the number of subjects available for analysis.

The limitations in Paper IV were that only 40 % of patients were warfarin naïve at baseline which affects TTR measurements. Secondly, a majority of the patients were white (87 %) with a low percentage of Asians (2 %) and blacks (1 %) making the results not generalizable to these ethnic groups. Thirdly, no patients with severe renal impairment (CrCl < 30 mL/min) were included in the RE-LY study as it was an exclusion criterion. Fourthly, the genome-wide hits found in the study, have not yet been replicated in an independent cohort. And last, the sample size of the study was small when searching for genetic effects on clinical endpoints.

Future perspective

Clinical studies of pharmacogenetic dosing

The CoumaGen II Study

Until recently, pharmacogenetic studies comparing genotype guided dosing with standard care have been small and inconclusive.[101-103] In March 2012 the CoumaGen II study comparing pharmacogenetic dosing to standard clinical care was published.[104] Patients were randomized between two pharmacogenetic regimens (PG-1 and PG-2) and compared to a parallel control group using standard care. The PG-1 arm used a modified version of the IWPC model (Paper II), adding the effects of different INR target ranges and smoking in the following way; for those with target INR 2.5-3.5, the calculated dose was increased by 15 %; for those with target INR 1.5-2.5, the calculated dose was decreased by 10 %. For smokers, the calculated dose was increased by 10 %. On day 2 to 7 a dose alteration protocol algorithm was used, increasing or decreasing the dose based on the INR value. The PG-2 arm used the same pharmacogenetic dose prediction model as PG-1 with the exception that *CYP2C9* variant status was ignored for the first two days (*1/*1 was used). At day 4 or 5 a dose-revision algorithm, developed by the IWDRC, was used.[80] From day 8 and forward both arms used the validated INR-based Intermountain Healthcare Chronic Anticoagulation Clinic Protocol Algorithm.[102] The parallel standard-dosing patient control cohort was identified by a query of the medical records databases of the three participating hospitals. In the standard-dosing cohort patients were treated with standard care, however the INR-based dose-modification algorithm developed by Intermountain is generally recommended.[102] The primary outcomes of the CoumaGen II study were percent out-of-range (OOR) INR values at 1 and 3 months and TTR.

The total number of patients available for analysis was 245 for the PG-1 arm, 232 for PG-2 arm and 1,866 for the parallel standard care group. When comparing the two pharmacogenetic arms, PG-1 and PG-2, PG-2 was non-inferior but not superior to PG-1. The combined PG cohort was superior to the parallel controls with an overall increase in TTR of 11 % and 13 % within 1 month and 3 months respectively (Table 14). Further, there was a significant lower number of adverse events in the pharmacogenetic treatment arms compared to the standard care group (4.5 % vs. 9.4 %, $p < 0.001$).

Although being a landmark study for pharmacogenetics, the CoumaGen-II study has some significant drawbacks. Primarily the lack of randomization to pharmacogenetic and non-pharmacogenetic dosing raises questions.

Table 14. *Primary endpoint results for the CoumaGen-II study. Adapted from Anderson et al.*[104]

Study arm	N	OOR 30 days	OOR 3 months	TTR 30 days	TTR 3 months
PG-1	245	30.6 %	30.3 %	70.2 %	71.7 %
PG-2	232	31.8 %	30.3 %	67.5 %	70.8 %
PG-1 and PG-2	477	31.2 %	31.3 %	68.9 %	71.2 %
Standard care	1,866	41.5 %	42.3 %	58.4 %	58.6 %

OOR = out-of-range INR values

Ongoing clinical trials

At least five clinical trials on pharmacogenetic dosing are on the way. Two of the largest are the Clarification of Optimal Anticoagulation Through Genetics (COAG) trial in the US, and the European Pharmacogenetics of Anticoagulant Therapy study (EU-PACT).[105, 106]

The COAG study is a two-armed, double-blinded, randomized controlled trial that compares genotype based dosing with clinical guided dosing. Both treatment arms use a baseline dose-initiation model[33] and a dose refinement model after four or five days of warfarin therapy.[80] The aim is to include over 1200 patients that are expected to be on warfarin therapy for at least three months. The primary outcome of the study is TTR within the first four weeks of therapy.

The EU-PACT study is a two-armed, single-blinded, randomized controlled trial aimed to recruit almost 3000 patients commencing anticoagulation therapy with warfarin, acenocoumarol or phenprocoumon. In the intervention arm, patients are dosed according to a drug-specific dosing algorithm, which is based on genetic information and clinical data. For warfarin, 3-4 starting doses are calculated by combining the IWPC pharmacogenetic prediction model (Paper II) with an estimated accumulation index for warfarin (Examples in Paper V).[37] After the starting doses of warfarin, a pharmacogenetic dose revision algorithm is used.[80] In the other arm, control patients will are dosed without information about genotype. The follow-up period is 3 months. The primary aim of the study is to show whether pharmacogenetics improves anticoagulation therapy by increasing TTR.

Cost effectiveness of genotype driven dosing

The cost effectiveness of genotype driven dosing compared to standard clinical care relies on many factors. Among these factors are; cost of genotyping, increase in TTR, improved efficacy and safety in terms of clinical endpoints and lowering of the number of patient visits. Assuming genotype

guided dosing is clinically validated, the cost involved with genotyping is one of the most influential factors for its future use.[95]

In August, 2007, the FDA updated the label for Coumadin, the brand name for warfarin, to include genotype information and encourage, but not require, use of genetic information when initiating patients on warfarin.[20] This was one of the driving factors for clinical laboratories to investigate new rapid genotyping tests and point of care devices.[107] The cost involved with genotyping using these assays has been estimated at 70 $ – 400 $ per patient.[107] Using blood, without DNA extraction, these assays can successfully genotype one sample with high accuracy in 2-8 hours.[107, 108]

New alternatives to warfarin

NOAC alternatives to warfarin therapy have recently been introduced. They include the direct thrombin inhibitor dabigatran (RE-LY study)[5] and the factor Xa inhibitors rivoxaban (ROCKET AF study)[7] and apixaban (ARISTOTLE study).[6] These NOAC are proven non-inferior or even superior to warfarin. A meta-analysis of 50,578 patients, comparing all three NOAC alternatives with warfarin, revealed significantly decreased risks of stroke or systemic embolism (odds-ratio NOAC vs. warfarin 0.82 with 95 % CI 0.74 – 0.91) and death (odds-ratio NOAC vs. warfarin 0.88 with 95 % CI 0.82 – 0.95). The main driver for these benefits, appearing without any relation to INR control, was substantial reductions in hemorrhagic stroke and intracranial hemorrhage. Therefore these intracranial events might be a specific risk when using the more general anticoagulation with warfarin as compared to the specific coagulation factor inhibition provided by the NOACs. In the meta-analysis the risk of major bleeding was not statistically significant with the NOAC vs. warfarin (odds-ratio NOAC vs. warfarin 0.85 with 95 % CI 0.0.69 – 1.05).[109] However, concerning major bleeding there was a significant heterogeneity with higher rates of gastrointestinal bleeding with dabigatran 150 mg and rivaroxaban but in contrast a substantial around 30% reduction in major bleeding with apixaban and dabigatran 110 mg when compared to warfarin.

One weakness of these trials was that the TTR for the warfarin arms were low, 64 % in the RE-LY study, 55 % in the ROCKET study and 62 % in the ARISTOTLE study, showing a need for improvement of the warfarin therapy. Another aspect of these new drugs is that there currently is no antidote, and agents to reverse the effect are still under development.[110] Still so far the clinical usefulness of an antidote in the clinical setting is also under debate based on the considerably short half-life of the new agents in comparison to warfarin.

The cost effectiveness of dabigatran in AF compared to warfarin was estimated in a recent paper by Shah et al.[111] The results indicated that for AF patients dabigatran is more cost effective in patients with poor INR control

(TTR < 57.1 %) whereas warfarin is more cost effective in patients with excellent INR control (TTR > 72.6 %).

The pharmacogenetics of dabigatran has been investigated in Paper C where genetic variants affecting the plasma levels have been identified, possibly relating to clinical events.

Future perspective - summary

The CoumaGen-II study is a large step towards the recommendation to genotype of *CYP2C9* and *VKORC1* when initiating a patient on warfarin.[104] The other well designed, large ongoing clinical studies will provide further information on the clinical utility and validity of personalized warfarin therapy. The future of pharmacogenetic dosing of warfarin relies on proper estimates of the improvement, in terms of efficacy and safety, of warfarin treatment using pharmacogenetic models compared to standard care, the clinical utility of the point of care testing, the availability and cost of genotyping and the cost-effectiveness compared with new oral anticoagulants.[95, 111, 112]

Conclusions

- Through candidate gene studies and GWAS we have shown that *CYP2C9* and *VKORC1* are the major genetic determinants of warfarin maintenance dose.
- A pharmacogenetic prediction model for warfarin maintenance dose, called the IWPC model, was developed including clinical factors and genotypes from *CYP2C9* and *VKORC1*. The predictive performance was excellent, with R^2 values ranging from 43 % to 51 %.
- The IWPC model performs better than a clinical prediction model (without genetic factors) with the largest benefit in patients requiring doses < 29 mg/week and ≥ 49 mg/week.
- The variation in predictive performance between ethnicities of the IWPC model, in terms of R^2, is mainly caused by differences in allele frequencies of the *VKORC1* SNP rs9923231.
- Other genes affecting maintenance dose, the replicated *CYP4F2* gene as well as the novel genes *NEDD4* and *DDHD1* found in the GWAS of this thesis, have small effects and are not likely to be important.
- Genotypes of *VKORC1* and *CYP2C9* affect TTR measures within three months and in total, as well as time to INR > 4.0 after initiation of treatment, indicating that pharmacogenetic dosing has the potential to improve efficacy and safety of warfarin treatment.
- The future of pharmacogenetic warfarin dosing relies on results from the ongoing studies, the availability of inexpensive genotyping and the cost-effectiveness of pharmacogenetic driven warfarin dosing compared with new oral anticoagulant drugs.

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