MAJOR DETERMINANTS OF OUTCOME AND DOSING IN WARFARIN TREATMENT

Jonatan Lindh

Stockholm 2009
– Whenever a theory appears to you as the only possible one, take this as a sign that you have neither understood the theory nor the problem which it was intended to solve.

Karl Popper
ABSTRACT

The aim of this thesis was to identify factors that determine individual patients’ sensitivity to the anticoagulant warfarin, and to quantify the effect of such factors on different measures of anticoagulation control. As part of this project, a large bio-bank was prospectively collected, with DNA and clinical data from more than 1500 patients starting warfarin treatment (the WARG cohort). To facilitate data retrieval and monitoring in this multi-centre trial, we developed two internet-based study interfaces, a browser-based protocol for manual data entry and a protocol for automated data extraction from an existing medical record system. All data was stored in a central database and monitoring of the data was performed in real-time via a browser-based monitoring interface. The Internet-based study tools proved efficient and safe, with a potential to improve quality and cost-effectiveness of future multi-centre studies.

In the WARG cohort, the incidence of first-time severe bleeding (according to the WHO criteria for severe adverse drug reactions) was 2.3 per 100 patient-years. Male gender and use of drugs potentially interacting with warfarin both increased the risk of severe bleeding, with odds ratios of 2.8 and 2.3, respectively. The incidence of severe bleeding was 2.4 times higher during the first month of treatment, compared to any time hereafter.

To evaluate the influence of genetic factors on the outcome of warfarin treatment, we analysed the DNA samples from the WARG cohort. Out of 29 polymorphic genes analysed, only two were clearly associated with warfarin dose requirements and anticoagulation control. These genes were VKORC1 (coding for warfarin’s target molecule), and CYP2C9 (important for the elimination of S-warfarin). During initiation of therapy, polymorphisms in VKORC1 and CYP2C9 increased the risk of over-anticoagulation significantly. The effect was most pronounced in the individuals homozygous for the CYP2C9*3 allele, with a hazard ratio of 21.8. The corresponding hazard ratio in patients homozygous for the VKORC1 haplotype A was 4.6. Carriers of VKORC1 haplotype A also reached therapeutic levels of anticoagulation more rapidly than others. An extended analysis of the association between CYP2C9 genotype and the risk of over-anticoagulation showed that the association was strong during the first two weeks of warfarin therapy, but abolished by the third week. Both VKORC1 and CYP2C9 genotypes were nominally associated with anticoagulation stability, measured as time spent within the therapeutic INR interval. However, this association was no longer significant after statistical correction for multiple testing.

The association between CYP2C9 genotype and warfarin dose requirements was further investigated in a meta-analysis, pooling data from 39 published studies to provide precise estimates of the gene-dose effect. Compared to the wild-type genotype (CYP2C9*1/*1), individuals with the *1/*2 genotype required doses that were 19.6% lower. Corresponding values were 33.7% for the *1/*3 genotype, 36.0% for *2/*2, 56.7% for *2/*3, and 78.1% for *3/*3.

Although warfarin has been on the market for more than 50 years, the knowledge of the drug’s effects in clinical use is still expanding, as demonstrated in our studies.
LIST OF PUBLICATIONS

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


# CONTENTS

1 Introduction ................................................................. 1
  1.1 History of warfarin .................................................. 1
  1.2 Pharmacology of warfarin ........................................ 3
    1.2.1 Molecular properties ........................................ 4
    1.2.2 Pharmacodynamics ........................................... 4
    1.2.3 Pharmacokinetics ............................................. 7
    1.2.4 Drug interactions ............................................ 10
    1.2.5 Monitoring of warfarin effect ............................. 11
  1.3 Warfarin bleedings .................................................. 12
    1.3.1 Observational studies ....................................... 12
    1.3.2 Randomised studies ......................................... 14
    1.3.3 Factors influencing bleeding risk ....................... 15
  1.4 Pharmacogenetics of warfarin .................................... 16
    1.4.1 CYP2C9 ......................................................... 18
    1.4.2 VKORC1 ......................................................... 20
    1.4.3 Other genes .................................................. 22
    1.4.4 Clinical application of warfarin pharmacogenetics .... 23
2 Aims ................................................................................. 26
3 Methods and results ....................................................... 27
  3.1 Patients ....................................................................... 27
  3.2 Data collection .......................................................... 27
  3.3 Genotyping .................................................................. 28
  3.4 Statistical methods ..................................................... 29
  3.5 Paper I ......................................................................... 30
    3.5.1 Study design ....................................................... 30
    3.5.2 Results ............................................................... 30
  3.6 Paper II ......................................................................... 31
    3.6.1 Study design ....................................................... 31
    3.6.2 Results ............................................................... 31
  3.7 Paper III ....................................................................... 32
    3.7.1 Study design ....................................................... 32
    3.7.2 Results ............................................................... 32
  3.8 Paper IV ....................................................................... 33
    3.8.1 Study design ....................................................... 33
    3.8.2 Results ............................................................... 33
  3.9 Paper V ....................................................................... 34
    3.9.1 Study design ....................................................... 34
    3.9.2 Results ............................................................... 34
4 Discussion ......................................................................... 35
5 Acknowledgements .......................................................... 42
6 References ........................................................................ 43
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DDD</td>
<td>Defined daily dose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EPHX</td>
<td>Epoxide hydrolase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>GGCX</td>
<td>$\gamma$-glutamyl carboxylase</td>
</tr>
<tr>
<td>INR</td>
<td>International normalized ratio</td>
</tr>
<tr>
<td>IRP</td>
<td>International reference preparation</td>
</tr>
<tr>
<td>ISI</td>
<td>International sensitivity index</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionisation – time of flight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPAF</td>
<td>Stroke prevention in atrial fibrillation</td>
</tr>
<tr>
<td>SPORTIF</td>
<td>Stroke prevention using an oral thrombin inhibitor in atrial fibrillation</td>
</tr>
<tr>
<td>THRIVE</td>
<td>Thrombin inhibitor in venous thromboembolism</td>
</tr>
<tr>
<td>$V_D$</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>VKOR</td>
<td>Vitamin K epoxide reductase</td>
</tr>
<tr>
<td>VKORC1</td>
<td>Vitamin K epoxide reductase subcomplex 1</td>
</tr>
<tr>
<td>WARG</td>
<td>Warfarin genetics</td>
</tr>
<tr>
<td>6-FAM</td>
<td>6-carboxy-fluorescein</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 HISTORY OF WARFARIN

A blizzard was blowing when Ed Carlson left his Wisconsin farm for a 300 km ride to Minnesota and the office of the State Veterinarian. Manoeuvring through the snow with a truckload consisting of a dead cow, a can of blood and 50 kg of spoiled sweet clover he was yet unaware that he was about to set off a chain of events that would ultimately save the lives of millions, while taking the lives of many, many others\[1, 2\]. In that winter of 1933, he had already lost five of his cows to spontaneous bleedings, and now the bull was oozing blood from the nose. Carlson was determined to find out what was causing him this disaster\[2\].

The dying cattle were not a complete mystery to Carlson. Similar outbreaks had been observed in the Wisconsin area since the 1920’s and two veterinarians, Schofield and Roderick, had established that the cause of the bleedings was to be found in improperly cured hay made from sweet clover\[3\]. However, Carlson had uneventfully fed his animals spoiled sweet clover before and was hesitant to accept that his farm was now haunted by the “sweet clover disease”. In addition, the prospect of having to replace the winter’s supply of hay may have contributed to the hesitance of a farmer suffering from the effects of a long-standing economic depression.

Hoping for an alternative explanation, Carlson was dismayed to find the State Veterinarian’s office closed for the weekend. By chance he ended up at the nearby department of Biochemistry at the University of Wisconsin where he was introduced to Professor Karl Link, the very same Link that would later be remembered for the discovery of the oral anticoagulants dicumarol and warfarin. At the time, he could only recommend Carlson to discard the spoiled hay, but according to Link’s own recollections the unfortunate fate of the farmer prompted Link and his co-workers to take on the quest for the anticoagulant agent in spoiled sweet clover. Six years later, in 1939, they succeeded in isolating dicumarol and within a few years the new drug’s antithrombotic potential had been explored in a large number of clinical trials\[2\].

In parallel with dicumarol’s introduction in clinical medicine, Link’s group synthesized hundreds of related coumarins with varying pharmacological properties. One of those substances, number 42, was selected as a candidate for use as rodenticide in a project sponsored by the Wisconsin Alumni Research Foundation*\[4\]. The initial letters of the sponsor combined with “arin” (from coumarin) gave the substance its official name, warfarin. Being a highly potent, water-soluble anticoagulant without discernable taste or odour, it was easily delivered via cereal grain in doses high enough to cause lethal internal bleedings in the exposed rodents. Unlike previously used rat poisons, warfarin had a slow onset of action (albeit faster than its progenitor dicumarol) and killed only after multiple doses\[2\]. This proved effective, since it masked the causal association between intake and injury, thus preventing the development of bait refusal or bait shyness in the rodent colonies\[4, 5\]. Commercially, it was an instant success and within a decade more than 60 000 000 kg of warfarin had been distributed in the United States alone\[2\].

* Ironically, 42 is also the correct answer to the ultimate question of life, the universe, and everything, given to the owners of the earth, i.e. the mice, in Douglas Adam’s novel “The Hitchhikers Guide to the Galaxy”\[6\].
Although the original oral anticoagulant dicumarol was an effective means of preventing thrombosis, researchers in the United States and Europe were continuously searching for more potent coumarins with faster onset of action, and a wider selection of administration routes. Since these properties were largely similar to those desired in a rodenticide, it is not entirely surprising that Link in 1950 proposed that warfarin should be tried as an antithrombotic[2]. Equally unsurprising was the initial reluctance among clinicians to use warfarin in patients, given the fact that the substance was already a well-established rodenticide[2]. However, it’s reputation as a poisonous drug was somewhat mitigated in 1951, after a report of an army inductee failing to commit suicide by ingesting a packet of rat poison containing more than 500 mg of warfarin[7]. In 1955, the drug’s reputation was further improved as the US president Eisenhower was given warfarin following a myocardial infarction and within a year it was considered “the anticoagulant of choice” in US hospitals[8]. Its popularity has prevailed, and today it is the most widely used anticoagulant in the world[9].

Initially, warfarin was primarily given for thrombosis prevention in patients with myocardial infarction, cardiac failure, venous thrombosis and pulmonary embolism[7], but the indications have widened over the years, as has the willingness to treat elderly patients. Today, the drug is also used in conditions such as chronic atrial fibrillation, cardiomyopathy, arterial and cardiac valve transplants, and for prophylaxis in e.g. electrical cardioversion or immobilisation[10]. In 2007, 1.6% of the Swedish population were treated with warfarin[11, 12] and the increased use is reflected in the Swedish drug sales statistics (figure 1) demonstrating a ten-fold increase in warfarin sales (adjusted for population size) in the last three decades[13]. Similar increases in warfarin use have been observed internationally, and in e.g. the United States the number of warfarin prescriptions dispensed annually increased from 21 to 30 millions between 1998 and 2004[14].

![Figure 1](image-url)  
*Figure 1. Total sales of warfarin tablets in Sweden 1971-2007, expressed as defined daily doses (DDD) per 1000 citizens per day. DDD is a standardised unit of measurement, defined as the assumed average maintenance dose per day for a drug used for its main indication in adults. Drugs are assigned DDDs by the WHO Collaborating Centre for Drug Statistics Methodology, and for warfarin the DDD has been set to 7.5 mg.*
1.2 PHARMACOLOGY OF WARFARIN

Pharmacology can be defined as the study of the effects of chemical substances on the function of living systems[15]. From a pharmacological point of view, warfarin is a fascinating drug. Its complicated elimination, mechanism of action, propensity for drug interactions, its tendency to cause severe bleedings in therapeutic use, and the strong influence of genetic factors on the individual sensitivity to its anticoagulant effects make it a powerful example of several important pharmacological principles. Unfortunately, the very same properties make it an extraordinarily difficult drug to manage clinically, requiring close monitoring coupled with clinical experience and a good deal of theoretical knowledge.

The desired effect of warfarin treatment is to reduce the risk of thromboses, by inhibition of the coagulation system. Since the physiological role of this system is to limit the clinical consequences of spontaneous and traumatic bleedings, it is not surprising that warfarin increases the risk of severe haemorrhage in a dose-dependent manner. To achieve a favorable balance between the protection from thrombosis and the risk of bleeding, it is essential to identify a dose that is neither too low nor too high. This task is troublesome for three main reasons. Firstly, warfarin – and other coumarins – have unusually narrow therapeutic intervals, i.e. the dose required for an adequate antithrombotic effect is close to the dose levels associated with drastically increased bleeding risk. Secondly, the dose required for a safe and effective anticoagulation differs widely between individuals. While some patients tolerate doses no higher than 0.5 mg per day, others require 30 or, in rare cases, more than 100 mg per day for effective anticoagulation[16]. Thirdly, there are no clinical signs of the dose being too low or too high, except for the occurrence of thromboses or bleedings. Hence it is not possible to rely on the observable clinical effect when adjusting the dose to the patient’s individual dose requirement. Rather, the dose adjustment has to be guided by repeated laboratory measures of anticoagulation intensity (see section 1.2.5).
1.2.1 Molecular properties

Warfarin is usually administered as a sodium salt, but in circulating warfarin a hydroxyl group has been exchanged for the sodium and the molecule consists of 19 carbon, 16 hydrogen, and 4 oxygen atoms, arranged in a 3-ring structure (figure 2)[16]. Warfarin has a molar mass of 308.3, typical of orally administered drugs[16, 17]. It is highly water-soluble and a weak acid with a pKₐ of 5.0[18].

![S-warfarin and R-warfarin](image)

**Figure 2.** Warfarin (R)- and (S)-enantiomers. The asterisks denote the chiral centre of each molecule.

A pharmacologically important aspect of the warfarin molecule is its chirality. The word “chiral” stems from the Greek noun *cheir*, “hand”, and refers to molecules that, like the hands, exist in two mirror versions. These mirroring images are called enantiomers, from the Greek word *enantio*, “opposite”[19]. The basis for warfarin’s chirality is the carbon atom abridging the molecule’s ring structures. This carbon simultaneously binds to one hydrogen and three different substituents and since the binding sites on the carbon atom cannot freely change places, the attachments can be arranged into two distinct mirroring patterns, giving rise to two warfarin enantiomers (S-warfarin and R-warfarin)[20]. Although structurally similar, the two enantiomers have different pharmacokinetic* and pharmacodynamic† properties, and interact with each other on both levels. Since warfarin is normally administered as a racemate, i.e. a 1:1 mixture of S-warfarin and R-warfarin, one might say that warfarin is not one but two drugs used together, further increasing the complexity of warfarin pharmacology.

1.2.2 Pharmacodynamics

Warfarin, and related coumarins such as acenocoumarol, dicoumarol and phenprocoumon, are commonly described as vitamin K antagonists as they exert their anticoagulant effect via interactions with vitamin K, necessary for normal blood coagulation[21].

---

* Pharmacokinetics describe the fate of drug molecules administered to a living organism, i.e. the absorption, distribution, metabolism, and subsequent excretion of the drug.
† Pharmacodynamics describe the effects of a drug at its site of action, i.e. interactions with drug receptors in the body and the events elicited by these interactions.
Vitamin K was first described in the early 1930s by the Danish biochemist Henrik Dam, who noticed that chicken fed a fat- and cholesterol-free diet developed spontaneous haemorrhage. Dam postulated the existence of a fat-soluble vitamin K (for Koagulation), and by exposing hundreds of chicken to different experimental diets he concluded that the substance was abundant in hog liver fat and hemp seeds. Within a few years other researchers had managed to purify the vitamin and determine its molecular structure, and in 1943 Dam and Edward Doisy were awarded the Nobel Prize in medicine for their work on vitamin K[22].

The important role of vitamin K (or actually its reduced form, vitamin K hydroquinone) is to act as an electron donor to the enzyme γ-glutamyl carboxylase (GGCX). In humans, this enzyme carboxylates at least fourteen different proteins, thereby making them biologically active. Since these carboxylations cannot take place in the absence of vitamin K, the substrates of GGCX are collectively known as vitamin K-dependent proteins. Four of the vitamin K-dependent proteins are circulating coagulation factors (factors II, VII, IX, and X) necessary for normal blood clotting. When newly synthesized in the liver cells, they are all inactive, but before they are secreted into the blood stream, they are activated by GGCX, enabling them to take part in blood coagulation when called to duty[23]. Vitamin K is also necessary for the activation of three anticoagulant proteins (protein C, S, and Z) that counteract blood clotting. Although the function of these proteins is also hampered in states of vitamin K deficiency, the effects on coagulation factors II, VII, IX and X is of larger importance and the net effect is a reduced ability to form blood clots. Even though vitamin K is mainly known for (and named after) its important effects on blood coagulation, vitamin K-dependent proteins are also involved in e.g. bone mineralization, apoptosis (programmed cell death) and growth control. Hence, warfarin and other vitamin K antagonists could potentially have effects outside the coagulation system[22]. For example, it has been suggested that long-term warfarin treatment could reduce the risk of malignancies[24].

When coumarins were first introduced as anticoagulants in the 1940s, it was already well-known that they acted by antagonising vitamin K and that their anticoagulant effects could be reversed by massive doses of this vitamin. Nevertheless, it was not until the 1970s the mechanism behind warfarin’s vitamin K-antagonizing effects was revealed. One clue to the mechanism is provided by the fact that the body’s content of vitamin K is very limited and the dietary intake of vitamin K ought not be sufficient to supply GGCX with the amount of electrons needed for the necessary carboxylation of coagulation factors and other proteins[22, 25]. The explanation for this apparent discrepancy is that vitamin K is not really spent after delivering its electrons to GGCX, a process that converts the vitamin from its reduced hydroquinone form to its oxidized form, vitamin K epoxide. Rather, the vitamin works like a rechargeable battery that, after being reloaded (i.e. reduced to vitamin K hydroquinone), is once again ready to power the carboxylation processes of GGCX. This repeated oxidation and reduction of vitamin K is known as the vitamin K cycle, and theoretically it could go on eternally. In practice however, the average vitamin K molecule spends only about 1.5 days in the body, before it is eliminated via the urine or the bile[22, 25]. Hence, a continuous intake of vitamin K is necessary to maintain adequate coagulation[26].
The reactivation of vitamin K does not occur spontaneously, but is mediated by an enzyme complex known as vitamin K epoxide reductase (VKOR). In the rechargeable battery analogy presented above, VKOR would be the battery charger, reducing the inactive vitamin K epoxide in two steps to active vitamin K hydroquinone (figure 3). VKOR is central to the understanding of warfarin and other coumarin anticoagulants, since they all act by binding to the enzyme complex and stopping it from reducing the vitamin K epoxide[23].

Figure 3. The vitamin K cycle. GGCX = γ-glutamyl carboxylas, VKOR = vitamin K epoxide reductase, VitKH2 = vitamin K hydroquinone, Vit KO = vitamin K epoxide, Vit KH = vitamin K.

Once at least 70% of the VKOR molecules in the liver have been inactivated, the GGCX runs short of vitamin K hydroquinone, and when GGCX can no longer keep up the carboxylation of newly synthesized vitamin K dependent coagulation proteins, these factors are secreted to the blood in their uncarboxylated, inactive form[23, 27, 28]. Initially, there are plenty of previously synthesized active coagulation factors in the blood, and the clotting ability is not severely affected. However, there is a constant turnover of coagulation factors and as the active ones are lost and replenished by inactive ones, the coagulation capacity decreases. The turnover time varies between different factors and while the plasma concentrations of active factor VII and IX have stabilized on low levels within three days after starting warfarin treatment, the levels of factor II and X keep declining for at least a week[29]. In addition, the levels of the vitamin K-dependent anticoagulant protein C decreases rapidly following initiation of warfarin, causing a paradoxical procoagulant state with increased risk of thrombosis during the first days of treatment[30]. To counteract this procoagulant effect, it is customary to initially combine warfarin with a parenteral immediate-acting heparin-type anticoagulant.

* Parenteral, from Greek para (beside) and enteron (intestine), refers to drug administration via routes not involving the gut, e.g. by injection into veins, muscles or skin.
† Heparins are immediate-acting anticoagulants that inactivate circulating coagulation factors II and X in the blood.
To complicate things further, the two warfarin enantiomers (S- and R-warfarin) are not equally effective inhibitors of VKOR. To achieve a similar inhibition of VKOR, R-warfarin plasma levels 3 to 5 times higher than those of S-warfarin are required, indicating that S-warfarin is a 3 to 5 times more potent inhibitor of the enzyme complex[31, 32]. However, these figures are based on experiments where the two enantiomers are given separately and when administered together (as is common practice), the anticoagulant effect seems to be attributable almost entirely to S-warfarin[27, 33]. In addition, in vitro experiments on liver microsomes indicate that the two warfarin enantiomers may actually be equally potent inhibitors once they reach VKOR, and that the divergent potencies seen in vivo could reflect differences in disposition and accumulation within the liver cells[34].

Once warfarin reaches VKOR, it binds very tightly to the molecule[35, 36]. Hence, fluctuations in plasma warfarin concentration over the dosing interval are not accompanied by similar changes in VKOR binding, making it difficult to establish a concentration-effect relationship based on plasma samples. The concentration-effect relationship is further obscured by the fact that warfarin’s direct pharmacologic effect (inactivation of VKOR) is only indirectly associated with the observable anticoagulant effect – also influenced by other factors such as genetic disposition, drug interactions and dietary intake of vitamin K[37].

### 1.2.3 Pharmacokinetics

Following oral intake of warfarin, the drug is rapidly absorbed from the stomach and the upper gastrointestinal tract, reaching maximal blood concentrations within 60-90 minutes[28]. The bio-availability (the fraction of administered drug that reaches the systemic circulation in unchanged form) is almost 100%, indicating a lack of significant pre-systemic metabolism[38].

Once in the systemic circulation, warfarin’s distribution to other tissues is very limited as indicated by a small volume of distribution, approximately 10 liters in an individual weighing 70 kg[39]. The volume of distribution ($V_D$) is a theoretical volume, defined as the ratio between the total amount of drug in the body at a specific timepoint and the drug concentration in plasma (or blood) at the same time. Since the numerator is a drug amount and the denominator a drug amount per volume, the unit of the resulting ratio is a volume. Although this volume does not refer to any actual body fluid or space (some drugs have $V_D$s of several thousand litres, well beyond the entire body volume), it provides a measure of a drug’s propensity to remain in the plasma, rather than being distributed to other parts of the body. This is of interest, since the body’s systems for elimination of drugs usually only target drug residing in plasma. Hence, drugs with a smaller $V_D$ tend to be cleared more rapidly, all other factors equal.

---

*Pre-systemic or “first-pass” metabolism refers to drug metabolism that takes place during the drug’s journey from the gut lumen to the systemic blood circulation, via the gut wall and the liver.*
Like many other acidic drugs, warfarin binds to albumin, a protein abundant in plasma. The interaction is such, that 97-99.5% of all warfarin present in plasma is bound to albumin, and this high degree of protein binding is likely to contribute to warfarin’s small V\textsubscript{D}[16, 40]. In vitro, the two enantiomers compete for the binding sites on human albumin. The competition seems to be asymmetric, with S-warfarin being able to completely displace R-warfarin, while even high concentrations of R-warfarin only displace a minor part of the albumin-bound S-warfarin[41]. Usually, only the unbound drug fraction is considered available for hepatic uptake, but there is controversial evidence that the uptake of some drugs (including warfarin) may be facilitated by albumin binding[42]. Since both the pharmacologic action of warfarin and its elimination through metabolism take place within the liver cell, enantiomeric competition for albumin binding could theoretically have an effect on the elimination rate of the two warfarin enantiomers, as well as on their relative contributions to the overall anticoagulant effect.

Warfarin is eliminated through liver metabolism, but the liver enzymes mediating the metabolism differ completely between the two warfarin enantiomers. While S-warfarin is almost exclusively metabolized by the polymorphic cytochrome P450 2C9 (CYP2C9) enzyme, R-warfarin is metabolized by a wide range of cytochrome P450 enzymes, including CYP3A4, CYP2C19 and CYP1A2 (but not CYP2C9)[43-46]. Following transformation into a number of hydroxylated or reduced water-soluble metabolites, warfarin is excreted in urine (80%) and faeces (20%), while the excretion of non-metabolized warfarin is negligible[21]. The elimination half-lives* differ between S-warfarin (18-35 hours) and R-warfarin (20-60 hours)[47]. Given warfarin’s small V\textsubscript{D} and the large fraction of drug residing in plasma where it is available for hepatic uptake, these half-lives are surprisingly long. The explanation for this is warfarin’s very low plasma clearance of only 0.2 L/h[39]. Clearance could be described as “the volume of blood (or plasma) from which all the drug would appear to be removed per unit time”[48]. It is proportional to the volume of distribution and inversely proportional to the elimination half-life, and is considered a primary measure of the body’s capacity to eliminate a drug[48]. Warfarin’s clearance is approximately one tenth of those of many other commonly used drugs[39]. Despite this low clearance, the metabolising enzymes involved seem to have a considerable capacity and linear† kinetics are preserved even after huge doses of 200 to 1000 mg[38]. Being a low-clearance drug, warfarin elimination is relatively insensitive to alterations in hepatic blood flow[39]. On the other hand, its low hepatic extraction fraction‡ makes it vulnerable to drug interactions based on liver enzyme inhibition[49].

* The elimination half-life of a drug is the time required for the drug concentration in plasma to decrease by 50%. After one half-life, the concentration is 50%, after two it is 25%, after three it is 12.5%, etc. The half-life influences the duration of drug effect and the dosage regimen (i.e. how often the drug must be administered to maintain a therapeutic effect).

† Linear, or first-order, kinetics refers to a situation when a constant fraction rather than amount of drug in plasma is eliminated per time unit. This is the common situation in drug therapy, and a requirement for the definition of an elimination half-life.

‡ The fraction of drug in plasma that is eliminated during a single passage through the liver.
For some drugs, especially those with a large volume of distribution and a long elimination half-life, it is customary to initiate the treatment with a “loading dose”, considerably larger than the doses given during maintenance treatment. The reason for this is that the plasma concentrations for such drugs increase only slowly if treatment is initiated with the calculated maintenance dose, since the time required to reach a stable plasma concentration is proportional to the elimination half-life. By giving a loading dose high enough to result in the desired maintenance concentration, it is theoretically possible to reach therapeutic concentrations from the very onset of treatment (figure 4).

Historically, warfarin therapy was initiated with loading doses of up to 1.5 mg/kg (approximately 20 times the average daily maintenance dose used today) because it was believed to provide therapeutic anticoagulation in the shortest period of time, but based on warfarin’s $V_D$, a loading dose of 15 mg (approximately 2-3 times the average maintenance dose) should be sufficient[39, 50]. As mentioned above, the anticoagulant effect of warfarin is delayed not only by a slowly ascending plasma concentration but also by a slow turnover of coagulation factors, reducing the potential benefit of a loading dose (see section 1.2.2). Results from randomized studies comparing warfarin starting doses 5 mg vs 10 mg have been diverging, and the optimal warfarin starting dose is still a matter of controversy[50].

**Figure 4.** Drug concentration in plasma with and without an initial loading dose on day 0 (onset of treatment). From the 2nd dose onwards, doses are identical in the two groups.
1.2.4 Drug interactions

The term drug interaction refers to a situation where the effect of a drug is altered by another drug, an herbal medicine or by food. The interaction can result in either increased or decreased pharmacologic effect of the drug, as well as an altered risk of specific side-effects. Commonly, drug interactions are divided into two groups, the pharmacokinetic and the pharmacodynamic interactions. The former group consists of interactions modifying the uptake, distribution and elimination of a drug, thereby altering the drug concentrations at the pharmacologic site of action (i.e. the drug target). The interactions in the latter group have no effect on drug concentrations, but instead alter the sensitivity to a specific drug concentration (i.e. the concentration-effect relationship) by actions on the drug target itself or on the chain of events coupling the drug target to the observable drug effect.

Drug interactions involving warfarin are exceedingly common. For example, a widely used reference book on drug interactions lists more than 500 warfarin drug interactions – a larger number than for any other of the reviewed drugs[51]. One reason for this abundance is of course warfarin’s narrow therapeutic interval, where even minor changes in drug concentration or drug sensitivity can have major clinical consequences. Another factor that is likely to be of importance is the elimination of S-warfarin being heavily dependent on a single liver enzyme (CYP2C9), making it vulnerable to pharmacokinetic drug interactions targeting this pathway.

The wealth of documented warfarin interactions involves a number of different pharmacokinetic and pharmacodynamic mechanisms. Some drugs, e.g. the lipid-lowering agent cholestyramine, reduce the absorption of warfarin in the gut, thereby attenuating its anticoagulant effect. Drugs that bind extensively to albumin may displace warfarin from its binding sites on the plasma protein, thereby increasing the unbound concentration and the anticoagulant effect[52], but the effect is only transient and rarely of clinical importance. The most important pharmacokinetic interaction mechanism is altered hepatic metabolism of warfarin due to inhibition or induction of cytochrome P450 enzymes, CYP2C9 in particular. Important inhibitors of warfarin metabolism include e.g. the antiarrhythmic drug amiodarone and severalazole-type antifungals, while inducers such as the anti-tuberculosis agent rifampicin and several anti-epileptic drugs may vastly increase the warfarin dose required for adequate anticoagulant effect. Pharmacodynamic warfarin interactions include drugs and food containing vitamin K or altering the turnover of vitamin K, and antithrombotic drugs such as aspirin, that increases the risk of bleeding by inhibition of platelet aggregation in the blood[53, 54].

The knowledge of warfarin interactions is largely based on case reports and small experimental studies, while the overall clinical importance of such interactions for the risk of adverse treatment outcome is less well understood. However, data from the UK General Practice Research Database indicate a 4-fold increased risk of severe bleedings in patients concomitantly treated with warfarin and antiplatelet drugs, as compared to warfarin alone. In the same study, use of other drugs potentially increasing the warfarin effect increased the risk of severe bleeding more than 3-fold[55]. Although drug interactions are likely to contribute significantly to the overall bleeding risk, the...
prescribers’ knowledge about these interactions is limited and prescription of potentially interacting drugs is common in warfarin-treated patients[56, 57]. Hence, aids such as computerised prescribing systems with automated detection of drug interactions could potentially improve the safety of warfarin treatment[58].

1.2.5 Monitoring of warfarin effect

When dicumarol was introduced as an anticoagulant, it was soon recognised that observing the clinical effect (i.e. the occurrence of thromboses or bleedings) was an insufficient means of assuring patient safety. Fortunately, a laboratory analysis suited for monitoring of coumarin effect was already available – the prothrombin time (PT) test originally described by Armand Quick in 1935[59]. Although the method has been subsequently modified, the basic principle remains the same. Firstly, calcium (necessary for normal coagulation) is immediately removed from a plasma sample to prevent it from clotting before the analysis is performed*. In the analysis, a strong procoagulant (“thromboplastin”†) is added to the sample together with calcium to set off the coagulation cascade, and the time required for clotting is measured. This time, the “prothrombin time”, was initially thought to reflect the functional activity of coagulation factor II (also known as prothrombin, hence the name “prothrombin time”) in plasma, but it was later realised that it simultaneously measures the activity of factor I, V, and three of the coagulation factors inhibited by coumarins, II, VII, and X. By addition of vitamin K-independent coagulation factors to the reagent, the method selectively measures the activity of factors II, VII, and X[60].

Originally, the thromboplastin was extracted from human brains in each laboratory but in the 1950s commercial supplies of thromboplastin derived from various animal tissues became available and were widely used. Much later, in the 1980s, it was realised that the choice of thromboplastin had a profound impact on the test results achieved. One clue to this was the fact that much higher warfarin doses were prescribed in North America (where commercial thromboplastins were widely used) than in the UK (largely relying on human brain thromboplastin) and when human brain and rabbit thromboplastins were compared in a randomised trial, it turned out that use of the latter caused five times more bleedings, despite a shorter target PT (i.e. lower anticoagulation intensity)[59, 61].

Acknowledging the need for uniform PT measurements, the World Health Organization in 1983 adopted international standard for reporting of PT, the international normalized ratio (INR)[62]. The INR system relies on an “international reference preparation” (IRP), a reference thromboplastin stored in the Netherlands. Every thromboplastin used anywhere in the world has been meticulously compared against the IRP, resulting in an “international sensitivity index” (ISI) that characterises the individual batch of thromboplastin. When patient samples have been analysed, the

---

* The blood sample is drawn into a test tube containing citrate, which binds to calcium.
† Thromboplastin (or “tissue factor”) has previously been known as coagulation factor III. Since it is no longer considered a coagulation factor, there is a numeric gap in the coagulation cascade. Likewise, factor IV now answers to the name of calcium.
ISI of the utilised thromboplastin is used for adjustment of the results, making them comparable to those achieved with other thromboplastins. Apart from the ISI correction, the INR is simply the ratio of the analysed sample’s PT and the average PT from a group of healthy individuals not taking anticoagulants[59]. Hence, an INR of 1 is equivalent to no anticoagulant effect, while an anticoagulant effect resulting in a doubling of the normal baseline PT gives an INR of 2.

The standardised INR made it possible to develop internationally accepted guidelines about proper anticoagulation intensity, and warfarin treatment today usually aims at maintaining an INR between 2 and 3. To accomplish this, the INR is measured repeatedly in warfarin-treated patients, and the dose is adjusted until therapeutic INR levels have been achieved. When treatment is initiated, there is a large uncertainty regarding the individual dose requirements and to avoid prolonged over- or underdosing, the INR should be analysed every 1-2 days. As confidence in the titrated dose increases, the intervals between INR controls are gradually increased. However, as dose requirements can change during the course of treatment (e.g. due to drug interactions or diseases) INR monitoring must continue as long as the patient is on anticoagulants and the intervals between measurements should rarely exceed 4-6 weeks[10].

1.3 WARFARIN BLEEDINGS

Warfarin prevents thrombosis by inhibiting the blood’s ability to coagulate. Hence, it is not surprising that bleeding is the most common side effect of the drug. Although warfarin-related haemorrhages are usually clinically minor (e.g. nose-bleeds and bruising), major intracranial, gastrointestinal and genitourinary bleedings are important deterrents to more widespread use of coumarins[63, 64].

Unfortunately, the anticoagulation intensity that effectively prevents thrombosis is dangerously close to that which causes bleeding in the warfarin-treated patient. Since such bleedings are sometimes disabling or even lethal, it is crucial that the unavoidable risk of haemorrhage is clearly outweighed by the beneficial antithrombotic effects in each patient where warfarin treatment is contemplated. This risk-benefit analysis requires not only correct estimates of warfarin’s beneficial antithrombotic effects, but also of its unwanted effects on the risk of severe bleedings. Regrettfully, information on the risk of warfarin-associated haemorrhage is conflicting, to say the least.

1.3.1 Observational studies

Since the risk predictions are to be applied to real-life patients, observational studies from routine medical service would be expected to offer the most valid estimates. From such studies we learn that the risk of a major bleeding in a patient treated with warfarin for one year is somewhere between 0.3% and 18%, information that hardly enables us to make a reliable risk-benefit analysis[65-79].

* The geometric mean, to be precise
The intensity of anticoagulant therapy is strongly associated to the risk of bleeding[80], and this could have contributed to the diverging results from different observational studies. Over the years, the targeted intensity of warfarin treatment has gradually been reduced, as a result of the INR standardisation and clinical studies indicating a better balance between thrombosis and bleeding risk in the INR range 2-3, as compared to higher target INRs. Hence, bleeding rates derived from older studies may not be relevant to today’s patients that are almost uniformly treated with the intention of maintaining an INR between 2 and 3.

Another important reason for the variation in risk of major bleeding is the way such events are defined in different studies. While a mere need for medical evaluation of the haemorrhage has been sufficient grounds for a diagnosis of major bleeding in some studies[70], others have required life-threatening events such as intracranial haemorrhage, cardiac tamponade or severe blood loss[66]. It is obvious that such differences in bleeding definition could have an impact on the haemorrhage risk estimate, and the importance of bleeding definition has been confirmed in a systematic comparison of three commonly used definitions of major bleeding[81].

Even if observational warfarin studies are expected to offer bleeding risk estimates that are applicable to ordinary patients subjected to routine medical care, they commonly share a weakness in lacking a control group. Theoretically, it would be possible to compare the bleeding incidence in the studied patients to that of a set of community controls with similar risk factors for bleeding, but without exposure to warfarin. However, this is rarely done since the information about patients, risk factors and bleeding events usually are extracted from medical records at anticoagulation clinics (where everyone for obvious reasons is exposed to anticoagulants). To find unexposed controls one would have to look among healthy individuals or patients in other kinds of clinics, where risk factors and bleeding events are likely to be less well-documented. Consequently, it would very difficult to decide whether an observed difference in bleeding risk between warfarin-exposed and unexposed individuals reflects a pharmacological effect of warfarin, or is merely the result of under-reporting in the unexposed individuals.

Bearing this in mind, results from observational studies with unexposed controls could still be of interest. One such study from 1999 included more than 21 000 patients with deep-venous thrombosis who were admitted to hospital and treated with warfarin[82]. During a period of three months following discharge from hospital, 1.4% of the patients were readmitted for bleeding, equivalent to an incidence rate of 5.6 bleedings per 100 patient-years. In a control group 39 000 matched patients with cellulitis or pneumonia (presumably not treated with anticoagulants), the 3-month incidence of readmission for bleeding was 0.7% (2.8 per 100 patient-years), half of that in the warfarin-exposed group. A more recent study from 2006 followed a 13 500 patients with atrial fibrillation for 2.4 years and compared the incidence of admission to hospital for bleeding in those who were on warfarin and those who were not[83]. The incidence turned out to be identical in the two groups, 1.1 per 100 patient-years, although intracranial haemorrhage was more common in the warfarin-treated patients. Another recent study identified patients with atrial fibrillation via the ECG (electrocardiogram) departments in hospitals and contacted the patients at six-month intervals with inquiries for recent
bleedings[84]. The incidence of major bleedings was approximately 2 per 100 patient-years regardless if the patient was treated with warfarin or not. However, the smaller size of this study (425 patients) rendered it a lower statistical power.

1.3.2 Randomised studies

In randomised studies, the patients are randomly allocated to one of two treatments that are to be compared. The virtue of the randomisation is that it effectively equalises all factors other than the treatment itself, thus reducing the risk of biased results. In randomised studies where warfarin is compared to placebo, it is therefore relatively safe to conclude that any difference in bleeding rates between the two groups is attributable to the warfarin treatment itself. The main drawback of randomised studies is that their validity in real-life patients is often questionable. For statistical and economical reasons, the patients included in randomised studies are often meticulously selected according to a number of inclusion and exclusion criteria, resulting in study populations that may have little in common with ordinary patients. In addition, the drug exposure is usually better monitored and the follow-up more ambitious than in clinical practise, factors that could result in overly optimistic estimates of bleeding rates.

Since randomised studies are often dimensioned to detect effects on thrombosis or a composite of thrombosis and bleeding, they frequently lack statistical power to estimate the incidence of less common outcomes such as major bleeding. Furthermore, randomised controlled trials of warfarin versus placebo are distinctly rare today, since most new anticoagulation studies aim at comparing newly developed anticoagulants to standard care, which often involves warfarin. Since it would be unethical to randomise patients requiring anticoagulation to placebo, these studies cannot tell us how many of the observed bleedings that are attributable to warfarin exposure and how many that would have occurred without anticoagulation. Another aspect of newer randomised anticoagulant studies is that they frequently recruit patients among those who are already on warfarin treatment, and let some of them switch to the new drug investigated. Since the risk of bleeding is highest in the early phases of warfarin treatment, such studies in already stabilised patients could under-estimate the bleeding risk[85].

Randomised studies that have actually compared warfarin to placebo include those performed to determine the optimum duration of anticoagulant treatment. A Swedish study by Schulman et al compared six weeks with six months of anticoagulation (warfarin or dicumarol) after a first episode of venous thromboembolism. The six-month risk of major bleeding was 0.2% (equivalent to 0.4 per 100 patient-years) in the six-week group and 1.1% (2.2 per 100 patient-years) in the six-months group[86]. However, the actual number of major bleedings was small (six) and the difference between the two groups was not statistically significant. Another study by the same author compared six months of anticoagulant therapy with anticoagulation continued indefinitely in patients who had had a second episode of venous thromboembolism[87]. During a follow-up time of four years, the incidence of major bleeding was 0.7 per 100 patient-years among those treated for six months and most of those bleedings occurred after the therapy was finished. In the group treated indefinitely, the incidence of major
bleeding was 2.2 per 100 patient-years, but again the number of bleedings was small (thirteen) and the difference statistically non-significant.

When turning to randomised studies without a placebo group, valuable information can be extracted from several large multicentre studies comparing the direct thrombin inhibitor ximelagatran* with warfarin, each providing data from more than a thousand warfarin-exposed patients. In two studies aiming at preventing stroke in patients with atrial fibrillation (SPORTIF III and SPRORTIF V), the incidence of major bleeding in warfarin-treated patients was 2.2 and 3.4 percent per 100 patient-years, respectively[88, 89]. A study in patients with deep-venous thrombosis (THRIVE) showed a higher incidence, 4.4 per 100 patient-years[90]. The fact that most patients in SPORTIF III and V were already stabilised on warfarin at the time of randomisation could have contributed to the lower frequencies in these studies. In an earlier study comparing warfarin to a combination of low-dose warfarin and aspirin in patients with atrial fibrillation (SPAF III), the incidence of major bleeding in the warfarin-only group was 2.1 per 100 patient-years[91]. As in the SPORTIF studies, most patients in SPAF III were stabilised on warfarin prior to inclusion.

1.3.3 Factors influencing bleeding risk

The intensity of anticoagulant therapy is an important determinant of haemorrhage risk during warfarin treatment and the risk of bleeding appears to increase exponentially with a linear increase in anticoagulant effect (INR)[64, 80]. Strong associations between INR level and the risk of bleeding have been reported in patients treated with warfarin for various indications[61, 92-95] and in randomised comparisons of anticoagulant intensities, patients assigned to INR-levels >3.0 experience more than twice as many major bleedings as those randomised to an INR of 2.0 to 3.0[61, 92, 93]. In addition, unstable INR levels have been associated with an increased bleeding risk, independent of the mean INR level. This correlation probably reflects an increased frequency of marked INR elevations in patients with unstable anticoagulation intensity[70, 96].

A number of studies indicate that the risk of bleeding is higher during the first 1 to 3 months of warfarin treatment (induction phase), compared to later time periods (maintenance phase)[67, 68, 70, 72, 82, 98-100]. Generally, the incidence rates of major bleedings have been approximately doubled during warfarin induction in these studies[72, 82, 98], but 10-fold increases have been described[68]. In a meta-analysis of 33 studies, Linkins et al found an increased risk of intracranial haemorrhage during the first three months of anticoagulation, as compared to therapy after the first three months (1.48 vs 0.65 per 100 patient-years, respectively)[101]. One theoretical reason for the increased risk of haemorrhage during warfarin induction could be that warfarin rapidly induces bleeding in a large proportion of the patients predisposed to such events. If this

* The first orally administered alternative to the coumarins was launched by AstraZeneca in 2004. Two years later it was withdrawn from the market because of suspected liver toxicity.
† Acronyms are very popular in medical science, possibly because of their ability to evoke positive attitudes towards the studied drug via a mechanism known as “automatic attitude activation”[97].
is true, the lower incidence during the maintenance phase could reflect a patient population selected for its ability to tolerate warfarin. In addition, patients with unexpectedly low dose requirements may have contributed, since they could be prone to over-dosing by the standard doses given at the start of treatment, before the dose has been individualised by means of INR-guided dose adjustments.

Although some reasonably sized studies have failed to show an association between age and risk of warfarin bleedings[67, 70, 72], there is a wealth of evidence that this risk increases with advancing age[80]. For example, Landefeld et al[68] have demonstrated a three-fold increased risk of major bleedings in patients older than 65 years of age and Pengo et al[102] a six-fold risk increase in patients older than 75. The effect of age remains when restricting the analysis to older patients, as demonstrated by Hylek et al[85]. When comparing patients ≥80 years of age to patients aged 65 to 79, they found a three-fold increased risk in the former group. In a recent Swedish study, Wallvik et al[79] found that the risk of bleeding increased with age over a wide range of ages, with an odds ratio of 1.05 per year of age (equivalent to 1.63 per decade). The mechanism behind this association has not been established[103].

Another patient characteristic that predisposes for anticoagulant-associated bleedings is comorbid disease. Diseases that have been associated with an increased risk include hypertension[68], renal insufficiency[71, 72, 82], and malignancy[82, 100, 104]. Some studies indicate a higher risk of anticoagulant-associated bleedings in women compared to men[70, 82, 105-107], but others have failed to show such an association[67, 72, 104, 108]. In addition, concomitant use of medication interacting pharmacologically with warfarin could increase the risk of haemorrhage, as discussed above (section 1.2.4).

Finally, it seems plausible that erroneous drug intake would increase the risk of bleeding. Although the possible consequences of warfarin dosing errors are well known, the average patient fails to take warfarin as prescribed more than one out of five treatment days, indicating that poor compliance could be a major clinical problem[109].

1.4 PHARMACOGENETICS OF WARFARIN

Pharmacogenetics combines the two disciplines of pharmacology and genetics, by addressing genetically determined variability in how individuals respond to drugs. The term was coined in 1959 by the German geneticist Friedrich Vogel, but the fact that drugs can have unexpected effects in certain individuals had been known long before that[110]. For example, Classic Greece doctors were aware that fava beans could cause anaemia and jaundice in predisposed individuals, a reaction known as “favism”[111]. More than two millennia later the hereditary defect predisposing for favism and a similar reaction to quinine-based antimalarial drugs was identified, a lack of the enzyme glucose-6-phosphate dehydrogenase common in Mediterranean

*It has been speculated that this was the reason for the mathematician Pythagoras prohibiting his followers from eating fava beans, although Aristotle claims Pythagoras was primarily aggravated by the beans’ resemblance to genitalia and/or the gates of Hades[111].
populations[111-113]. In individuals with this enzyme defect, the erythrocytes are unable to maintain a supply of reduced glutathione necessary to cope with oxidative stress from exposure to e.g. fava beans and particular drugs and such exposure therefore causes a rapid destruction of the erythrocytes[111].

Although sporadic observations of hereditary differences in sensitivity to drugs had been made earlier, it was not until the 1950s that pharmacogenetics emerged as a distinct discipline. The development of new techniques for precise measurements of enzyme activities and drug concentrations was a driving force in the characterization of genetically determined drug acetylation and the identification of serum-cholinesterase deficiency causing prolonged muscle paralysis in patients treated with succinylcholine[110, 114]. Another pharmacogenetics milestone was the discovery of cytochrome P450 in the late 1950s[115, 116]. This liver-derived carbon monoxide binding pigment efficiently absorbing light with a wavelength of 450 nm (hence its name), subsequently turned out to be a large family of enzymes metabolising innumerable endogenous and exogenous substances, including a large number of commonly used drugs[116-118]. From twin studies, it was evident that genetic factors were of major importance for the individual ability to metabolise drugs[110, 119, 120]. The realisation that polymorphisms in the various cytochrome P450 genes leading to inactive or low-active enzymes could explain a sizable portion of this variability made the CYPs main targets for pharmacogenetics research. Initially, the scientific efforts focused on a single cytochrome P450 enzyme, CYP2D6, also known as debrisoquine or sparteine hydroxylase because its metabolic effects were first described in these two now obsolete drugs[110, 121, 122]. To date, more than 70 CYP2D6 polymorphisms have been described[123], many of which result in an inactive enzyme. Carriers of two such inactive alleles are commonly referred to as “poor metabolisers” and have an increased risk of adverse effects from drugs inactivated by CYP2D6[124]. In addition to the poor metabolisers and the “normal” extensive metabolisers, there are individuals with up to 12 extra copies of the active CYP2D6 gene[125]. These “ultrarapid metabolisers” are instead at risk of therapeutic failure and require substantially higher doses of common CYP2D6 substrates[126, 127]. Further research has established the importance of polymorphisms in other CYPs such as CYP1A2[128], CYP2C9[129, 130], CYP2C19[131], and CYP3A5[132] in treatment with a number of common drugs, including warfarin (see section 1.4.1). In addition to the cytochrome P450 system, polymorphisms of genes that encode drug transporters and drug targets have gained increasing research interest in later years[110] (see section 1.4.2).

* A drug target is a molecule (e.g. an enzyme, ion channel or hormone/neurotransmitter receptor) to which the drug binds, thereby altering its activity. This binding starts the chain of events that leads to the observable drug effect.
1.4.1 CYP2C9

The story of warfarin pharmacogenetics began with CYP2C9. In 1999, Guruprasad Aithal and co-workers showed that polymorphisms in the CYP2C9 gene were associated with reduced dose requirements in warfarin treated patients and that a low dose requirement was a risk factor for major bleeding [133]. This indirect connection between CYP2C9 genotype and warfarin-associated haemorrhage inspired massive scientific efforts in the area and for the last decade warfarin has been one of the most thoroughly investigated drugs in the field of pharmacogenetics.

Cytochrome P450 2C9 (CYP2C9) is located on chromosome 10, together with other members of the CYP2C subfamily (e.g. CYP2C8 and CYP2C19) [134]. It was first cloned in 1991, but the three-dimensional structure of the encoded enzyme was not resolved until 2003 [135]. CYP2C9 preferentially metabolises weakly acidic molecules such as non-steroidal anti-inflammatory drugs (NSAIDs), oral antidiabetics, and S-warfarin, the more potent of the two warfarin enantiomers [117, 136].

Within five years after the cloning of CYP2C9, two polymorphisms altering the metabolism of S-warfarin in vitro had been identified [137, 138]. These polymorphisms, CYP2C9*2 and CYP2C9*3, are relatively common among Caucasian populations, with allele frequencies of 11-16 and 7-10%, respectively [139]. They both cause exchanges of amino acids in the CYP2C9 enzyme molecule (an arginine to cysteine at position 144 and isoleucin to leucin at position 359, respectively). The altered enzymes are still functional, but the clearance of S-warfarin is reduced compared to that of the wild-type enzyme (CYP2C9*1) [137, 138]. From in vitro experiments, it is evident that CYP2C9*3 has a drastically reduced enzymatic affinity* for S-warfarin, in combination with a reduced maximum enzyme velocity (v_{max})† [137, 140-142]. The effect of CYP2C9*2 is also likely to involve a reduced v_{max}, but the enzyme affinity seems to be unaltered or even increased [137, 138, 143]. Experimental factors such as the levels of cytochrome P450 oxidoreductase (a protein necessary for cytochrome P450-mediated metabolism) could have a major impact on the results and their applicability in vivo is uncertain [143]. Nevertheless, studies in patients and healthy volunteers have confirmed that both CYP2C9*2 and CYP2C9*3 are associated with reduced clearance of S-warfarin [142, 144-150]. These studies indicate that clearance of S-warfarin is approximately 20 and 45% lower in individuals carrying one CYP2C9*2 or CYP2C9*3 allele, compared to wild-type (*1/*1) individuals. In homozygous CYP2C9*3 carriers clearance is reduced by 90%, indicating that they may achieve plasma levels of S-warfarin ten times higher than in wild-type patient given equal doses.

---

* Low-affinity enzymes bind less efficiently to their substrates (e.g. S-warfarin), often resulting in a lower rate of metabolism.
† The rate of metabolism increases with increasing substrate concentrations, but it cannot exceed v_{max}.

---

Those gifted with two different allelic versions are heterozygous.
Since warfarin doses are adjusted until therapeutic INR levels have been achieved, the
dose given after successful titration should represent the treated individual’s sensitivity
to warfarin. At least sixty studies have addressed the association between CYP2C9
genotype and warfarin dose requirements[37, 133, 142, 144-146, 148-201]. These
studies provide overwhelming evidence that CYP2C9 polymorphisms have an impact
on the response to warfarin, and that the effect of CYP2C9*3 is larger compared to that
of CYP2C9*2. However, the quantitative impact of specific CYP2C9 genotypes varies
widely between studies and since most the individual studies are relatively small the
uncommon genotypes are poorly represented. Frequently, the scarcity of genotypes
based on two variant alleles (e.g. *2/*2, *2/*3 or *3/*3) have prompted aggregation of
several genotypes in the analyses, reducing the predictive value of the results.

Compared to wild-type, the *1/*2 genotype has been associated with reductions in dose
requirements of 0-40%, while dose reductions of 0-70% have been observed in *1/*3
carriers. Individuals with two variant alleles have usually required dose reductions of
20-90%.

Since patients start their treatment with standard doses (sometimes adjusted according
to age, co-medication etc.) there is an obvious risk that patients with an unforeseeably
low dose requirement are over-dosed during warfarin induction, before the dose has
been adjusted in response to INR measurements. However, this is not the only
mechanism by which CYP2C9 polymorphisms could expose patients to an increased
risk of warfarin-associated bleedings. As mentioned in section 1.2.3, clearance is of
importance not only for the drug concentration achieved with a certain dose, but also
for the elimination half-life and hence the time required to reach a stable concentration
at the start of treatment or after dose adjustments. Since patients with variant CYP2C9
genotypes have a reduced S-warfarin clearance and react unusually slowly to warfarin,
the standard monitoring schedule may not be adequate for these individuals.

Theoretically, ill-timed monitoring could lead to premature re-adjustments of the
warfarin dose and failure to maintain INR within therapeutic levels over time. Although
this has not been extensively investigated, available data indicate that it takes longer to
achieve a stable level of anticoagulation in carriers of variant CYP2C9 alleles[161, 196]. Accordingly, some studies indicate that individuals with variant CYP2C9 alleles
spend less time within the therapeutic INR interval[167, 197], but other studies have
failed to show such an association[189, 202].

Several studies have shown that CYP2C9*2 and CYP2C9*3 increase the risk of over-
anticoagulation, measured as high INR levels[161, 180, 181, 183, 203, 204] and the
risk is likely to be particularly high in individuals carrying two variant alleles[183, 205, 206]. Elevated INR levels expose patients to an increased risk of haemorrhagic
complications and several retrospective studies have indicated a several-fold increased
risk of bleeding in individuals with one or more variant CYP2C9 alleles[161, 167, 168, 170, 179]. Others have failed to show an association between CYP2C9 genotype and
haemorrhage[164, 191] but the small number of bleedings observed in these studies
makes the results difficult to interpret.

In addition to CYP2C9*2 and CYP2C9*3, a number of other variant alleles have been
identified and the Human Cytochrome P450 Allele Nomenclature Committee now
acknowledges 33 variant CYP2C9 alleles, some of which alter the enzymatic
activity[123]. The importance of these alleles for the outcome of warfarin treatment has rarely been investigated, and compared to CYP2C9*2 and CYP2C9*3 they are uncommon, at least among Caucasians. In other populations, the distribution of CYP2C9 alleles differs. The CYP2C9*2 allele, for example, has not been observed in Asians and CYP2C9*5, CYP2C9*6† and CYP2C9*11 have almost exclusively been identified in individuals of African descent[141, 207, 208].

In addition to warfarin and other drugs, CYP2C9 metabolises endogenous substances such as arachidonic acid and linolenic acid. By altering the turnover of such substrates CYP2C9 polymorphisms could theoretically have effects unrelated to drug therapy[209] and studies have indicated that low-active CYP2C9 alleles may increase the risk of myocardial infarction and depression[210-212].

### 1.4.2 VKORC1

Although methods of measuring the biologic activity of vitamin K epoxide reductase (VKOR) have been available since the 1970s, its molecular structure remained unknown for more than three decades. However, in 2004 two research groups independently identified the gene coding for warfarin’s target molecule[213, 214]. The gene, located on chromosome 16, was named vitamin K epoxide reductase complex subunit 1 (VKORC1) to allow for the possibility that the VKOR enzyme complex could include yet unidentified proteins in addition to the VKORC1 gene product[22]. Because of the previous investigations on CYP2C9 and warfarin, several research groups were already in possession of DNA collections from patients with known outcome of warfarin therapy. Analysis of the VKORC1 gene in such samples contributed to a rapid exploration of the gene’s importance in warfarin therapy.

A number of polymorphisms have been discovered in both coding and non-coding regions of VKORC1, and several of these have been associated with altered warfarin dose requirements[152-154, 156, 157, 165, 166, 171-173, 176, 178, 182, 183, 195-199, 215-227]. However, a majority of the identified point mutations are in linkage disequilibrium†, making it difficult to discern the ones functionally responsible for the altered warfarin sensitivity. Based on ten common non-coding SNPs Rieder et al identified two main haplotype groups (set of SNPs that are inherited jointly), group A and group B[222]. Haplotype group A has been associated with a reduced VKORC1 gene expression and lower than average warfarin dose requirements while haplotype group B predisposes for high warfarin dose requirements[222, 228]. The impact of the VKORC1 genotype is such that homozygous haplotype A carriers (AA) require approximately 40 to 70 percent lower warfarin doses compared to BB carriers, while heterozygotes (AB) have an intermediate warfarin sensitivity[152-154, 156, 157, 165,

---

† Unlike CYP2C9*2 and CYP2C9*3 that encodes an active enzyme, albeit with reduced activity, CYP2C9*6 causes a frame shift in the DNA template resulting in a complete loss of enzyme activity.

‡Polymorphisms in linkage disequilibrium are located close to each other on the chromosome and are therefore inherited jointly more often than would be expected from chance. Since individuals carrying the first polymorphism tend to have the second one as well, both polymorphisms could seemingly be associated with a trait that is entirely caused by one of them.
Two SNPs, 1639G>A and 1173C>T are commonly used to identify the VKORC1 A haplotype. However, the scientific literature in the area is complicated by the parallel use of several different systems for annotating these and other VKORC1 polymorphisms. The relationship between three VKORC1 nomenclature systems commonly used in warfarin pharmacogenetic studies is presented in Table 1. As an alternative to the A and B haplotype groups, Geisen et al divided the VKORC1 variants into four main haplotypes, VKORC1*1, *2, *3 and *4[229]. VKORC1*1 is considered to be the ancestral haplotype and is common only in populations of African origin. VKORC1*2 is equivalent to haplotype group A, hence predisposing for a low warfarin dose requirement[230]. It is the predominant haplotype in Asian populations, but less common among Caucasians and Africans[225, 231]. VKORC1*3 and *4 are both included in the high-dose haplotype group B, but there is some evidence that the warfarin dose requirements could differ further between these two high-dose haplotypes[157, 217]. In addition to these relatively common VKORC1 variants, a number of rare mutations predisposing for warfarin resistance have been described[220, 232-234]. Such mutations were of great importance for the discovery of the VKORC1 gene in 2004[213].

### Table 1. Nomenclatures of VKORC1 polymorphisms.

<table>
<thead>
<tr>
<th>D’Andrea</th>
<th>dbSNP</th>
<th>GenBank</th>
<th>Nucl. exchange</th>
<th>Rieder A</th>
<th>B</th>
<th>*1</th>
<th>*2</th>
<th>*3</th>
<th>*4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1173</td>
<td>rs9934438</td>
<td>6484</td>
<td>C→T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1639</td>
<td>rs17878363</td>
<td>3673</td>
<td>G→A</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs9923231</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1542</td>
<td>rs8050894</td>
<td>6853</td>
<td>G→C</td>
<td></td>
<td></td>
<td>●</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4931</td>
<td>rs7196161</td>
<td>381</td>
<td>T→C</td>
<td></td>
<td></td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2255</td>
<td>rs2359612</td>
<td>7566</td>
<td>C→T</td>
<td>●</td>
<td></td>
<td></td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3730</td>
<td>rs7294</td>
<td>9041</td>
<td>G→A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>3462</td>
<td>rs7200749</td>
<td>8773</td>
<td>C→T</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>698</td>
<td>rs17708472</td>
<td>6009</td>
<td>C→T</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each row represents a single nucleotide polymorphism (SNP) analysed in pharmacogenetic studies and its association with the haplotypes defined by Rieder [222] and Geisen [229] is indicated by bullets. The positions of the SNPs are presented according to three nomenclature systems, D’Andrea [157], The Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/) Reference Cluster ID, and GenBank (accession number AY587020). The nucleotides are A = adenosine, C = cytosine, G = guanine, and T = thymine.

A low warfarin dose requirement due to VKORC1 polymorphisms makes the patient prone to over-anticoagulation by the standard doses given at the start of treatment. A number of studies have independently demonstrated a several-fold increased risk of over-anticoagulation (based on INR) in carriers of VKORC1 haplotype A[182, 183, 196, 202, 226, 235, 236]. Although this risk is most pronounced during the initial phase of anticoagulation, before the dose has been adjusted according to the INR.
measurements, there is some evidence that the VKORC1 haplotype could influence the risk of high INR values throughout the course of treatment[183, 226]. Although such high INR values should increase the risk of haemorrhage, an association between VKORC1 haplotype and bleeding risk has not yet been established[168, 195, 202].

An important difference between VKORC1 and CYP2C9 (section 1.4.1) is that the former has no effect on the half-life of warfarin. Hence, the effect of the VKORC1 haplotype on warfarin sensitivity is observable from the onset of treatment, while that of low-active CYP2C9 variants reveals itself slowly, as the warfarin concentrations increase over extended periods of time[33]. Accordingly, several studies have failed to show an effect of the VKORC1 haplotype on the time required to establish a stable maintenance dose[182, 202, 226]. Nevertheless, a Swedish study by Osman et al found that warfarin-treated patients homozygous for the VKORC1*2 haplotype had a higher percentage of INR values outside the therapeutic interval and required twice as many visits over a 12-month period, indicating difficulties to establish and maintain a correct maintenance dose[221].

The genetic regulation of VKORC1 expression is not confined to individuals treated with anticoagulants, and just like the CYP2C9 dittos, VKORC1 polymorphisms have been associated with altered disease risk. For example, homozygous carriers of the VKORC1*2 allele seems to have a substantially lower risk of venous thromboembolism, arterial vascular disease, and graft injury following renal transplantation, compared to individuals with other haplotype combinations[237-239], although other studies have failed to show an association between VKORC1 haplotype and thrombotic disease[240-242].

1.4.3 Other genes

In addition to CYP2C9 and VKORC1, a number of other genes could be of importance for the outcome of warfarin treatment[243]. For example, the GGCX (\(\gamma\)-glutamyl carboxylase) and EPHX1 (epoxide hydrolase) genes are both involved in the vitamin K cycle[243] and polymorphisms in these genes have been associated with altered warfarin dose requirements in a small number of studies [148, 198, 215, 244, 245]. Another gene of potential interest is APOE (apolipoprotein E), regulating the uptake of vitamin K into the liver[246, 247]. The APOE allele predisposing for the most effective uptake of vitamin K, \(\epsilon4\), has been associated with increased warfarin dose requirements[248], while an increased risk of warfarin bleedings has been observed in carriers of the low-uptake \(\epsilon2\) allele[249]. However, other studies have shown no association between APOE genotype and warfarin effect[250], or a paradoxically reduced vitamin K antagonist requirement and increased risk of over-anticoagulation in carriers of the \(\epsilon4\) allele[201, 251].

Several of the coagulation factors carboxylated by GGCX are themselves polymorphic, and mutations in the genes coding for factor II[148, 252], VII[148, 152, 252], IX[253, 254], and protein C[255] have been associated with altered warfarin effect. Other genes such as drug transporters, cytochrome P450 enzymes constituting minor pathways of warfarin metabolism, and modulators of the vitamin K cycle could theoretically be of
importance for the outcome of warfarin therapy, but this has not been substantiated by empirical evidence[232, 243]. Interestingly, an association study investigating the impact of polymorphisms in a panel of 170 genes coding for common drug metabolising enzymes and drug transporters failed to find any associations between warfarin dose and polymorphisms in genes transporting or metabolising warfarin, except for CYP2C9. However, a polymorphism in the cytochrome P450 gene CYP4F2 enzyme was significantly associated with warfarin dose requirements, explaining an additional 2% of the overall variability. CYP4F2 is not known to metabolise warfarin, but it could theoretically be involved in the hydroxylation of vitamin K[256]. A recent genome-wide association study† of genetic factors influencing warfarin dose requirements identified no genes other than CYP2C9 and VKORC1, but the small number of included patients gave the study a low power for detection of less influential dose determinants[257].

To summarise, polymorphisms in a number of genes could potentially modify the anticoagulant effect of warfarin, but this has yet been unambiguously shown only in the case of CYP2C9 and VKORC1. A common denominator of most studies in the field of warfarin pharmacogenetics is the retrospective recruitment of patients. Usually, patients have been recruited among those prevalently visiting an anticoagulation clinic and medical records have been used to recapture warfarin doses, INRs or adverse events from the past treatment. This method of recruitment and data capture is time-saving and economically advantageous, but it introduces a risk of bias since the patients receiving anticoagulation at any given time-point are likely to differ from those about to start warfarin therapy – the ones in whom genotyping has been proposed. For example, patients with lifelong warfarin therapy (e.g. recipients of mechanic heart valves) should be more common among patients on warfarin than among those commencing therapy, and patients who have had the unfortunate fate of dying from a warfarin-related bleeding will by definition be excluded from retrospective studies. To avoid such bias, it is important that the pharmacogenetics of warfarin is addressed in prospective studies recruiting patients at the very start of therapy.

1.4.4 Clinical application of warfarin pharmacogenetics

Although it is clear that pharmacogenetics are of importance for warfarin dose requirements, and is likely to influence the treatment outcome, it is not self-evident how this knowledge should be utilised to improve safety and effectiveness. A common proposal is that genotyping should be performed before warfarin therapy commences, and that the genetic information is used to calculate a suitable maintenance dose to be used from the very start of treatment. Individualising the dose in this fashion could potentially eliminate the iterative trial-and-error aspect of warfarin induction, thereby reducing the time required to achieve a therapeutic level of anticoagulation and the risk of bleedings due to excessive warfarin effect. A number of algorithms have been developed in different patient populations, for translation of CYP2C9 and VKORC1

---

† In a genome-wide association study hundreds of thousands of marker polymorphisms are genotyped, providing a map of the entire genomes of the studied individuals. This genetic information is combined with an observed trait (e.g. warfarin dose) to identify genes and polymorphisms of importance for this trait.
genotype information into predicted warfarin maintenance doses[154, 172, 173, 196-199, 215-217, 223, 258-262]. Polymorphisms in CYP2C9 and VKORC1 explain approximately 13 and 20% of the total interindividual variability in warfarin requirements (less in individuals of African descent[259, 262]), and by including additional factors such as age, weight, and interacting medication the algorithms have been able to account for almost two thirds of the total variability[154, 172, 173, 178, 195-199, 215-217, 223, 258-262].

Even though pharmacogenetic algorithms may successfully predict the dose requirements in warfarin-treated patients, this does not automatically imply that genetically tailored dosing will improve the outcome of anticoagulation. To prove this, the algorithms should ideally be prospectively validated in randomised studies comparing them to standard care. Yet, only three prospective controlled studies have addressed the use of pharmacogenetic dosing[151, 200, 263]. The study by Hillman et al was a small (48 patients) feasibility study aiming at validating the genotyping procedure itself[200]. Neither time within therapeutic INR range nor incidence of INR>4 was improved by CYP2C9 genotyping, but the study did not have the statistical power to assess effects on these outcomes. The study by Caraco et al included 191 patients that received warfarin doses according to a genetic or non-genetic algorithm from the start of treatment[263]. Those that had their warfarin dose tailored according to CYP2C9 genotype reached therapeutic INR levels and stable anticoagulation 2.7 and 18 days earlier, respectively. In addition, pharmacogenetically dosed patients spent more time within the therapeutic interval and experienced 74% fewer minor bleedings. The study by Anderson et al included 206 patients and used a pharmacogenetic algorithm based on CYP2C9 and VKORC1, incorporating loading doses on the first two treatment days[151]. It failed to show an effect on the primary outcome, time outside the therapeutic interval, but the pharmacogenetic algorithm resulted in fewer INR measurements and dose adjustments. In addition, an exploratory analysis restricted to patients with genotypes predisposing for unusually high or low dose requirements showed that these outliers benefited from pharmacogenetic dosing, eliminating one quarter of the time spent outside the therapeutic INR interval. The results from the prospective studies emphasize that the choice of study size, target population, pharmacogenetic dosing strategy, comparator therapy and effect measure could have profound impact on study outcome and further research is clearly needed to identify the optimal mode of individualising warfarin therapy.

In each of the three prospective studies, pharmacogenetic dosing was associated with fewer major bleedings, but the number of events was small (less than 10 in each study) and the differences non-significant. A meta-analysis of the three studies indicated that pharmacogenetic dosing reduced the risk of major bleeding by 32% during the first month of treatment (relative risk reduction), but this pooled effect was still not statistically significant[264].

Attempts have been made to quantify the economic cost-effectiveness of pharmacogenetic warfarin dosing[265, 266]. Although these calculations indicated that routine genotyping of Caucasians starting warfarin therapy is likely to be cost-effective, they were based on rather crude assumptions (e.g. that the entire bleeding risk attributable to CYP2C9 polymorphisms could be eliminated by genotyping) and new
pharmacoeconomic studies based on data from prospective studies are warranted. Nevertheless, the US Food and Drug Administration (FDA) have chosen to change the labelling of warfarin to include a recommendation about lower starting doses in carriers of variant CYP2C9 and VKORC1 alleles[267] and institutions such as The College of American Pathologists have advised the Centers for Medicare and Medicaid Services to reimburse the testing[268].
2 AIMS

The overall aim of this thesis was to determine the frequency of severe bleedings in Swedish warfarin-treated patients, and to identify risk factors that predispose individual patients for such bleedings. A special emphasis was placed on genetic factors modifying the outcome of warfarin treatment, with the ultimate goal of improving warfarin safety by means of genotype-based dose individualization. The specific aims of the individual studies were:

Study I: To develop and implement two Internet-based study tools for data acquisition in the Warfarin Genetics (WARG) study.

Study II: To determine the incidence rate of severe bleedings in Swedish warfarin-treated patients, and to identify non-genetic factors predisposing for such bleedings.

Study III: To investigate the influence of CYP2C9 polymorphisms on the risk of over-anticoagulation during the early phases of warfarin treatment.

Study IV: To investigate the influence of polymorphisms in 29 candidate genes on warfarin dose requirements, time to stable anticoagulation, time within therapeutic INR range, and risk of over-anticoagulation. An additional aim was to develop a warfarin dosing algorithm incorporating demographic, clinical and genetic factors.

Study V: To calculate precise estimates of the warfarin dose requirements in individuals with specific CYP2C9 genotypes.
3 METHODS AND RESULTS

3.1 PATIENTS

Patients included in studies I-IV all belong to same cohort, recruited within the framework of the WARG study. Patients were included in 40 Swedish clinics (39 of these were specialised anticoagulation clinics) between December 2001 and August 2005. Only warfarin-naïve adults starting warfarin treatment, regardless of indication, were eligible for inclusion. Out of 1542 included patients, 19 (1.2%) were subsequently excluded, leaving 1523 in the final cohort.

Studies I and III were performed before patient recruitment and data collection were completed, and are based on subsets (n=909 and n= 219, respectively) of the final cohort (n=1523). Study I included all patients recruited at the time, while study III only included patients who had finished their warfarin treatment.

The median age of the included patients was 66 years, and the majority (63%) was male. The main indications for anticoagulation with warfarin were atrial fibrillation (51%), deep venous thrombosis (25%), pulmonary embolism (12%), and artificial heart valve transplants (2.3%). Almost all included patients (97%) had a target INR of approximately 2.5.

In addition to the WARG cohort, study IV included 181 warfarin-treated patients from a previous study (validation cohort)[255]. These patients were recruited at the Uppsala University Hospital anticoagulation clinic in 2000. At the time of recruitment, they had been treated with warfarin for at least two months and had achieved a stable warfarin dose[191].

Study V, a meta-analysis, included studies where warfarin-treated patients with known warfarin dose requirements had been genotyped with regard to CYP2C9*1, *2 and *3. The meta-analysis had no restrictions concerning patient characteristics, indications for warfarin treatment, treatment intensity and duration, or definition of warfarin dose requirement. A total of 7907 patients from 39 studies (including the WARG cohort) were included in study V.

3.2 DATA COLLECTION

Data collected in studies II-IV (the WARG study) is presented in table 2. The information was registered by the nurses, biomedical analysts, and physicians administering warfarin, at the time of inclusion and in association with routine INR measurements. Data was entered either via a web-based study protocol or by automated data extraction from the medical record system used at 68% of the participating centres. Both these modes of data acquisition are described in paper I. In addition to the clinical information, a blood sample for DNA analysis was drawn at the time of inclusion and sent by mail to a central bio-bank at our coordinating centre at Karolinska university hospital (Huddinge site).
Table 2. Data collected in the WARG study (papers II-IV).

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Baseline</th>
<th>Every visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Gender</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Risk factors for bleeding/thrombosis</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Indication for warfarin therapy</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Target INR</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Concomitant medication</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>INR</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Warfarin doses</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Bleedings</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Thromboses</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Next scheduled visit</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>

In paper V data was collected by means of a structured literature review. Publications presenting data on warfarin dose requirements in patient groups defined according to CYP2C9 genotype was searched in PubMed and EMBASE, and identified articles were evaluated for eligibility by a single reviewer. Selected articles were re-evaluated by two independent reviewers, who also extracted data to be used in the meta-analysis. Extracted data included study population characteristics (age, gender, weight, body surface area, nationality, ethnicity, use of interacting drugs, indications for warfarin therapy, target INR) and warfarin maintenance dose in each of six CYP2C9 genotype groups based on the *1, *2, and *3 alleles.

3.3 GENOTYPING

The prospectively collected blood samples were used for the genotyping, performed retrospectively.

DNA was extracted from white blood cells in whole blood by use of the MagnaPure method (MagnaPure DNA Isolation Kit - Large Volume; Roche Diagnostics, Mannheim, Germany).

Genotyping of CYP2C9 (*2 and *3 alleles) was performed using TaqMan real time PCR with allele-specific VIC\textsuperscript{TM} and 6-FAM\textsuperscript{TM}-labeled probes (Applied Biosystems, CA, USA)[269-271].

All other SNPs (181 SNPs in 29 genes of possible importance for the individual sensitivity to warfarin) were genotyped with Homogenous Mass Extend and iPLEX assays (Sequenom, Hamburg, Germany) followed by MALDI-TOF mass spectrometry[255, 272].
3.4 STATISTICAL METHODS

Descriptive statistics included calculation of median and interquartile range, or total range.

Methods for between-group comparisons of continuous variables included analysis of variance (ANOVA) and the Kruskal-Wallis test. When the over-all test demonstrated a significant difference between the groups, a post-hoc Dunnet test or group-wise comparison of mean ranks was performed to elucidate the differences between individual groups (paper III).

Univariate and multivariate linear regressions were used to assess the influence of continuous and non-continuous predictors on continuous outcomes (papers II and IV), while conditional logistic regression was used in a matched case-control analysis to assess the influence of INR on bleeding risk (paper II). In the linear regressions, the coefficient of determination, $R^2$ was used to measure the proportion of explained variance. The predictive performance of the multiple linear regression model was cross validated by calculation of the $R^2$ distribution of 10 000 random samples from the training data set. In addition, it was validated in an independent data set (paper IV).

Proportions were compared by means of the Pearson Chi-square test and Fisher exact test (papers III and IV). For analysis of time to event (i.e. survival analysis) we used the log rank test (univariate analyses) and Cox proportional hazards regression (multivariate analyses) (papers II and IV).

The combination of effect estimates from several studies in paper V was done by means of multiple meta-analyses. The individual studies included in the meta-analyses were weighted according to inverse variance (with addition of a random effects variance component in the random-effects model). Homogeneity among studies was addressed by the Cochran Q test, and by calculation of I$^2$ (the variation across studies attributable to heterogeneity rather than chance). Possible sources of heterogeneity were further investigated in a weighted linear regression model (“meta-regression”).

All tests were double-sided, and p-values $<$0.05 were considered statistically significant. In study IV, this significance level was adjusted by means of Bonferroni correction for multiple testing based on the effective number of independent tests, calculated by a spectral decomposition method[273]. After correction for multiple testing, only p-values $<$0.00029 were considered statistically significant.
3.5 PAPER I

3.5.1 Study design

To facilitate the collection of data* in the WARG† study (paper II-IV), two internet-based study tools were developed. The first of these was a study protocol accessible via a standard web browser. The protocol included a number of functions for data quality control and information integrity was ascertained by password protections and encrypted data transfer. The second tool was a computer-to-computer interface for automated real-time extraction of clinical data from an existing computerised medical record system. Incoming data from both channels were pooled in a central study database, accessible for monitoring via a web-based monitoring interface. Education of participating clinicians was provided for by means of written instructions and telephone support. Paper I is a method article describing the two systems and preliminary experiences from their application in the WARG study. Hence, it does not involve formal hypothesis testing.

3.5.2 Results

After two years of data collection, 909 patients commencing warfarin treatment had been included at 39 centres, with a drop-out rate of 2.8%. Both systems for data collection proved efficient, although the time-savings associated with the automated data extraction interface made this method especially attractive for future applications. Although a test system environment was offered, most participants chose to enter real study data from start, without complications, and the average number of questions requiring telephone support was less than one per month.

The opportunity to monitor study data in real-time proved very useful, enabling the detection and elimination of potential problems at an early stage.

---

* Data included patient characteristics, warfarin doses, INR values, concomitant medication, and information about bleedings, thromboses and deaths. In addition, a DNA sample was collected from each patient at the time of inclusion.
† WARG is an acronym for WARfarin Genetics, a prospective cohort study of patients starting warfarin treatment.
3.6 PAPER II

3.6.1 Study design

The prospectively recruited WARG cohort was used to estimate the incidence rate of severe bleeding in first-time warfarin users. Bleedings were defined according to the WHO criteria for severe adverse drug reactions, requiring hospitalisation (or prolongation hereof), permanent injury or death. Secondary endpoints were all-cause mortality and a composite of severe bleeding or death. The potential influence of age, gender, average warfarin dose, target INR, time outside the therapeutic INR interval, and interacting drugs on these endpoints was investigated in a multivariate analysis. Additional analyses addressed the influence of time (relative to treatment start) and INR level on the risk of severe bleedings.

3.6.2 Results

The incidence of first-time severe bleeding was 2.3 per 100 patient-years (95% confidence interval 1.4; 3.1). When repeated bleedings were also included, the incidence was 2.6 per 100 patient-years (1.7; 3.5).

The incidence of severe bleeding or death was 4.3 per 100 patient-years (3.2; 5.5). The only significant risk factors for severe bleedings were male gender (odds ratio 2.8) and use of drugs potentially interacting with warfarin (OR 2.3). Target INR and time outside therapeutic INR interval were significant predictors of death, with odds ratios of 46 per INR unit and 1.4 per 10\textsuperscript{th} of time in study, respectively. Predictors of severe bleeding or death were male gender (OR 2.0) and time outside therapeutic INR interval (OR1.7).

The incidence of severe bleeding was 2.4 times higher during the first month of treatment, compared to any time thereafter. At the time of severe bleeding, INR in the afflicted patients was significantly higher (3.1) than in matched controls (2.5), indicating an influence of INR on the risk of bleeding.
3.7 PAPER III

3.7.1 Study design

A subset of patients from the prospectively recruited WARG cohort was used to investigate the association between CYP2C9 genotype on the risk of high INR values (INR>3 and INR>4), indicative of increased bleeding risk. For these analyses, patients were divided into three groups according to CYP2C9 genotype: *1 (homozygous *1 carriers), *2 (*1/*2 and *2/*2), and *3 (any genotype containing the *3 allele). The analyses were done separately for treatment weeks 1, 2, and 3, to assess the time-dependency of the CYP2C9 effect.

3.7.2 Results

During the first treatment week the risk of achieving an INR>3 was 2.8 (95% confidence interval 1.2; 6.7) and 6.1 (2.7; 13.6) times higher in the *2 and *3 groups, respectively (using *1 as reference). During the second week, the relative risks were lower, 2.1 (1.2; 3.7) in the *2 group and 3.5 (2.1; 5.8) in the *3 group. By the third week, no influence of CYP2C9 on the risk of INR>3 was observed, with risk ratios of 1.0 to 1.1.

Analysis of the secondary endpoint INR>4 showed even greater risk increases (figure 5), but the small number of qualifying events rendered the analysis a low statistical power and only the tenfold increased risk seen in *3 carriers during the second week was statistically significant. Compared to the CYP2C9 effect on INR >3, the influence on INR >4 was seemingly delayed, with a risk peak during the second week and point estimates of the relative risk still exceeding 1 during the third week.

Figure 5. Relative risk of an INR >4 during treatment week 1-3 in the CYP2C9*2 and CYP2C9*3 groups. The CYP2C9*1 group is used as reference.
3.8 PAPER IV
3.8.1 Study design

The DNA samples collected in the WARG study (1496 samples) were used for genotyping of 183 SNPs in 29 polymorphic genes of possible importance for the individual sensitivity to warfarin. The genes chosen for analysis were involved in e.g. the distribution and metabolism of warfarin, the vitamin K turnover, and the synthesis and activation of coagulation factors and endogenous anticoagulant factors. A previous pilot study had associated polymorphisms in several of these genes with altered warfarin dose requirements[255]. The genotype information was tested for association with warfarin dose requirements and an algorithm for prediction of individual warfarin dose requirements was developed. The algorithm was based on both non-genetic and genetic factors found to be of importance for the sensitivity to warfarin and the final model was validated in 181 patients from a previous study[255]. In addition, the genotype data was tested for association with secondary outcomes, including time to stable anticoagulation, time spent within the therapeutic INR range, over-anticoagulation (INR>4), and bleeding.

3.8.2 Results

After correction for multiple testing, polymorphisms in VKORC1 and three cytochrome P450 genes, CYP2C8, CYP2C9 and CYP2C19, were associated with the warfarin dose requirements. However, the polymorphisms in CYP2C8 and CYP2C19 were almost entirely attributed to linkage disequilibrium with CYP2C9*. The prediction model developed included polymorphisms in VKORC1 and CYP2C9, age, gender, and interacting drugs. It explained 58.1% of the total variance in dose (52.8% in the validation cohort).

None of the analysed genes were significantly associated with time to stable anticoagulation. Before correction for multiple testing, both VKORC1 haplotype and CYP2C9 genotype were significantly (p<0.00029) associated with time within the therapeutic INR interval. Individuals with the low-dose VKORC1 AA haplotype were within range 70% of the time compared to 64% in the BB carriers. These associations did not remain significant after correction for multiple testing.

Polymorphisms in both VKORC1 and CYP2C9 were associated with INR>4 during the first five weeks of treatment. The highest risk was seen in CYP2C9*3/*3 carriers with a hazard ratio of 21.8 (compared to CYP2C9*1/*1). No polymorphism was significantly associated with bleeding, but one out of eight CYP2C9*3/*3 carriers (12.5%) experienced a serious bleeding during the first five weeks compared with 0.27% of patients with other genotypes.

* CYP2C8, CYP2C9 and CYP2C19 are all located in a “CYP2C” cluster on chromosome 10. When non-influential polymorphisms in CYP2C8 or CYP2C19 are inherited together with CYP2C9 polymorphisms altering warfarin dose requirements the effect of the latter can erroneously be attributed to the polymorphisms in CYP2C8 and CYP2C19.
3.9 PAPER V

3.9.1 Study design

A systematic literature search was performed to identify studies providing data on warfarin dose requirements associated with specific CYP2C9 genotypes. If required, the authors were contacted for additional information. After adjustment for between-study differences not attributable to CYP2C9 genotype, dose data from the individual studies were pooled in five separate meta-analyses. Using the homozygous wild-type (*1/*1) genotype as reference, each meta-analysis provided an estimate of the relative dose reduction associated with each of the five variant genotypes *1/*2, *1/*3, *2/*2, *2/*3, and *3/*3. Sensitivity analyses were performed to estimate the influence of age, ethnicity, interacting drugs, and target INR on the gene-dose association.

3.9.2 Results

Out of 1171 studies initially identified, 39 were included in the final analysis. Twenty of these could be included only after retrieval of additional data from the authors. A total of 7907 patients were included in the five meta-analyses. The dose reductions associated with individual CYP2C9 genotypes ranged from 19.6% (*1/*2) to 78.1% (*3/*3). The effect of each genotype on dose requirements are presented in figure 6. None of the factors investigated in the sensitivity analyses had a significant modifying effect on the association between CYP2C9 genotype and warfarin dose requirements. However, the impact of CYP2C9 genotype tended to be larger in studies where drugs interacting with warfarin were not allowed compared to studies where patients using such drugs had been included.

![Figure 6](image_url)

Figure 6. Reductions in warfarin dose requirements associated with individual CYP2C9 genotypes. Brackets denote 95% confidence intervals.
4 DISCUSSION

For a long time, prospective pharmacogenetic studies have been largely restricted to experiments in small groups of genetically characterised volunteers while the genetic influence on real-life clinical outcome has mainly been studied in retrospectively assembled groups of patients. Although these approaches are adequate for the discovery and preliminary exploration of gene-drug association, the demands for prospective evaluation of pharmacogenetic effects and the efficacy of personalized medicine are now increasing. Meeting these demands require larger and more costly studies, often with recruitment in several centres. Such multi-centre studies increase the complexity of data collection and monitoring. In paper I, we demonstrated the feasibility and usefulness of internet-based study protocols in a large prospective study set in 40 centres over a large geographic area.

For obvious reasons, internet-based study protocols are most advantageous in multi-centre studies. They are also attractive in studies gaining from a close monitoring in real-time, and in situations where interim analyses are required. However, their potential reaches well beyond decreasing the work-load on the monitoring facility. With implementation of automated data validation algorithms, the computerised study protocols can improve the formal and – to some extent – medical quality of the data collected and prepare the dataset for subsequent statistical analysis. After the WARG study (papers I-IV) was launched in 2001, the number of clinical trials employing internet-based protocols has increased and the technology is likely to be of even greater importance in the future. However, the automated data extraction from an existing medical record system that demonstrated advantages over the browser-based protocol in WARG has not yet become a mainstay mode of data acquisition in medical research. One explanation for this could be that the WARG study setting offered conditions usually not present in clinical trials. One such factor was the use of a single medical record system at a majority of the centres, eliminating the need for multiple computer system interfaces. An even more important factor was the structured medical records used to document anticoagulation, with continuous structured recording of doses and INR values ideal for automated data extraction. Nevertheless, it is foreseeable that medical record system developed with data synthesis and export in mind will facilitate a wider user of automated data extraction in the future.

In paper II, we demonstrated an incidence of first-time serious bleeding in warfarin-treated patients amounting to 2.3 per 100 patient-years. Compared to older studies from the 1980s and 1990s, this incidence is relatively low, indicating that the safety of warfarin therapy has improved over time[65, 68, 70-73, 76]. There are several possible explanations for this development, including the introduction of specialised anticoagulation clinics and standardised INR measurements, lower starting doses, intensified follow-up and an increasing awareness of individual factors influencing drug response. In addition, the definition of severe bleeding could have influenced the results. We chose to adhere to the internationally accepted WHO definition of severe adverse drug reactions. Since these criteria are comparatively strict, requiring admission to hospital, prolongation of hospital stay or permanent injury, they may result in lower incidence estimates compared to alternative definitions with wider
inclusion criteria. At face value, the bleeding incidence observed in the WARG cohort is not considerably higher than that seen in recent randomised controlled trials, indicating that the results from such studies may be valid in routine clinical care[88-90]. In addition, the relatively low incidence of severe bleedings could have implications for the potential benefit of pharmacogenetically tailored warfarin doses. If genotyping is mainly perceived as a means of preventing bleedings in patients predisposed for such events, a low frequency of bleedings reduces the potential gains of pharmacogenetics. However, there are alternative ways to look at the value of personalised medicine, as will be discussed below.

Of the non-genetic potential risk factors for bleeding analysed in paper II, only drugs interacting with warfarin and male gender had significant effects on the primary outcome, the incidence of major bleeding. Although pharmacologic interactions between warfarin and individual drugs have been well-documented in observational and experimental settings, few studies have been able to associate the full range of potentially interacting drugs with elevated bleeding risk. Our results underscore the clinical importance of drug interactions in warfarin therapy and make an important contribution to the understanding of anticoagulation safety. The increased bleeding incidence in men is surprising, since previous studies have usually found the opposite[70, 82, 105-107]. Although the over-risk in male patients could represent e.g. gender-specific patterns of alcohol intake or co-morbidities, it may also be a chance finding.

In papers III-V we investigated several aspects of warfarin pharmacogenetics. The meta-analysis in paper V pooled published data from a large number of pharmacogenetic studies to provide precise estimates of the association between CYP2C9 genotype and warfarin dose requirements. Such estimates are valuable for reference purposes, for pharmacokinetic/pharmacodynamic modelling and for dose recommendations in individual patients. For example, the prospective study by Caraco et al determined the warfarin doses by using a non-genetic dosing algorithm and multiplying the calculated dose with a correction factor defined individually for each CYP2C9 genotype[263]. Such factors could easily be derived from the results presented in paper V. Another interesting finding in this paper was that studies allowing the use of drugs interacting with warfarin showed a smaller impact of CYP2C9 genotype on the warfarin dose requirements. This effect was seen in the two largest genotype groups (CYP2C9*1/*2 and CYP2C9*1/*3) as well as in the CYP2C9*2/*2 group. Although the association was not statistically significant, it could indicate that drugs altering CYP2C9 activity have a potential to mask the influence of genetic polymorphisms on the enzyme activity. The study-level analyses performed in paper V have low power of detecting effects of patient characteristics such as concomitant medication, and it would definitely be interesting to investigate the potential interaction between genotype and interacting drugs in individual patients.

Whereas previous retrospective studies have indicated an increased risk of over-anticoagulation in individuals with variant CYP2C9 alleles[161, 181], paper III added information about the time-course of this association. In a prospective setting, we demonstrated that the CYP2C9*2 and CYP2C9*3 alleles were associated with a drastically increased risk of over-anticoagulation during the first two weeks of warfarin
treatment. However, by the third week there was no evidence of an influence by CYP2C9 genotype, indicating a successful dose titration guided by INR measurements. If this holds true, pre-treatment CYP2C9 genotyping has a clear potential to prevent bleedings during the first weeks of therapy, but may not improve safety during maintenance therapy. This notion is further supported by paper IV, where the CYP2C9 genotype had no effect on the over-all time within the therapeutic INR interval. If CYP2C9 genotyping would only prevent bleedings during the first two weeks, this limits the potential gain of the analysis, since only 12% of the major bleedings in WARG occurred within this timeframe. Nevertheless, other studies have associated variant CYP2C9 genotypes with increased bleeding risk well beyond the second week[161, 168] and it is not unlikely that genotyping and a personalised follow-up could increase safety during the maintenance phase.

When adding information about VKORC1 haplotype in paper IV, it was evident that this gene too was of importance for the risk of early over-anticoagulation. In addition, we demonstrated that carriers of the VKORC1 AA haplotype spent significantly more time within the therapeutic INR interval. This is a novel finding, although Schwarz et al have demonstrated an effect on the time above the therapeutic interval[183]. In their study, patients homozygous for VKORC1 haplotype A spent more time above the therapeutic interval, to some extent contradicting our findings.

The relative importance of CYP2C9 and VKORC1 for the outcome of warfarin treatment is of importance for the potential value of genotyping. In paper IV, polymorphisms in VKORC1 explained a larger proportion of the total variability in dose requirements (30%) compared to those in CYP2C9 (12%). However, the high explanatory value of VKORC1 is mainly attributable to the fact that the haplotype A allele was much more common (allele frequency 39%) than the CYP2C9*2 and CYP2C9*3 variants (allele frequencies 11% and 7%). Although the impact of the VKORC1 haplotype on the dose requirements is limited in individual patients, the abundance of each haplotype (A/A, A/B, and B/B) makes them quantitatively important for the overall variability. The CYP2C9 genotype on the other hand could have a much larger impact on the dose requirements in individual patients, while the relative scarcity of these alleles limit their influence on total dose variability. This is especially true in carriers of the CYP2C9*3/*3 genotype. These individuals demonstrate a dramatically exaggerated response to warfarin but since the genotype is only present in 0.5% of the patients it will not have a discernable effect on the total dose variability in the genotyped population. Consequently, genotyping of VKORC1 is definitely important to achieve correct warfarin dose predictions in the overall patient population, but genotyping of CYP2C9 could be of larger importance for the identification of a smaller number of high-risk patients.

Several genes other than CYP2C9 and VKORC1 have been associated with the outcome of warfarin therapy in previous studies. These, and a number of other genes, were analysed in paper IV, but none of them (possibly with the exception of CYP2C19) were found to be significantly associated to warfarin dose requirements or anticoagulation control. This emphasizes the need for confirmation of pharmacogenetic effects observed in small studies, to rule out the risk of chance findings and publication bias. It also verifies that the pharmacogenetic information implemented in warfarin
dosing algorithms could be restricted to CYP2C9 and VKORC1. Although other genes such as CYP4F2 could still be of some importance, they are unlikely to be major predictors of warfarin sensitivity[256, 274]. However, the potential contribution of additional genes will be further investigated in an ongoing genome-wide association study based on the WARG material.

One important question remains; should genotyping be routinely performed in patients starting warfarin therapy? Although intensive scientific efforts have been made in the field of warfarin pharmacogenetics during the last ten years, this question remains elusive and it may be better to approach it as a set of related questions:

1. Are genetic factors of importance for the outcome of warfarin therapy?

The answer to this question is clearly “yes”. Polymorphisms in VKORC1 and CYP2C9 are of major importance for the individual warfarin dose requirements and are likely to influence the stability of anticoagulation intensity and the risk of warfarin-associated bleeding.

2. How should pharmacogenetic information be used to individualise warfarin therapy?

The answer to this question is not quite as obvious. A number of algorithms (including one presented in paper IV) has been developed to translate patient-specific genetic and non-genetic factors into a predicted warfarin dose requirement[154, 172, 173, 196-199, 215-217, 223, 258-262]. Presumably, genotyping should be performed pre-treatment, allowing the patient start with a correct maintenance dose from the very beginning of treatment. However, this requires fast genotyping (currently not available at most hospitals) or the therapy will be delayed until the genotyping results arrive. In addition, it is unlikely that the predicted dose matches the dose requirements exactly and INR monitoring remains necessary. Once INR values are available, most algorithms give no guidance on how to adjust the dose taking both genotype and INR response into account. This problem has been addressed in two studies presenting “dose refinement” algorithms, offering dose prediction based on genotype information and the INR value obtained after three administered doses[173, 275]. These studies show that polymorphisms in both VKORC1 and CYP2C9 remain significant predictors of warfarin dose after taking the INR value into account. Due to the effect of CYP2C9 activity on the half-life of warfarin, patients with uncommon CYP2C9 genotype are likely to benefit from individualised monitoring schedules throughout the treatment[33, 146], but specific recommendations are still lacking. Another unanswered question is how to adjust the commonly used loading doses in accordance to genotype. Since loading doses are determined by V$_D$ rather than the elimination of a drug, it is not evident that the loading dose should be adjusted in the same manner as the maintenance dose. On the other hand, warfarin toxicity has been described after a single 10 mg dose in a patient homozygous for CYP2C9*3, indicating that regular loading doses may not be safely administered in these patients[205].
3. Can pharmacogenetics improve the outcome of warfarin therapy?

The information available to answer this question is scarce. Obviously, identifying a problem is not the same as solving it. Evaluation of a pharmacogenetic dosing strategy requires prospective studies with a control group where genotyping is not performed. Ideally, allocation to these groups should be randomised to avoid various sources of bias. The design of such studies involves several choices concerning e.g. the timing of genotyping, the dosing and monitoring strategies in both genotyping and control group, and the outcome measure. Obviously, there is a large number of ways in which these components can be combined and the three randomised studies published so far differ widely regarding these parameters[151, 200, 263]. The results from the three studies are presented in section 1.4.4, but some aspects deserve mentioning.

Anderson et al failed to show an effect of genotyping on the primary outcome (time outside the therapeutic INR interval) in the entire study population, but when the analysis was restricted to individuals with genotypes predisposing them to extreme dose requirements, genotyping did improve the outcome. Intuitively, it may seem logic to demand a positive effect in the over-all patient population in whom pharmacogenetic screening is proposed. However, a large proportion of these patients do not have genotypes associated with excess risk of bleeding and genotyping cannot be expected to reduce the risk of such events in these individuals. Obviously the costs associated with genotyping the low-risk individuals should be included in the total cost of a screening program, but when included in outcome analyses, they dilute the positive effects of genotyping in high-risk individuals and reduce the statistical power.

An analogous situation would be a screening program for early detection of a specific type of cancer. It would not be reasonable to demand that the screening program reduced over-all mortality in the entire screened population (analogous to any bleeding in warfarin-treated patients). Rather, one would probably be satisfied if the screening reduced the mortality attributable to the specific cancer screened for (analogous to bleedings in genetically predisposed patients). By a priori restricting the main analysis to the high-risk patients identified in the genetic screening, future studies could increase the chances of demonstrating an effect of genotyping in patients requiring warfarin therapy.

The research on warfarin pharmacogenetics has often focused on over-anticoagulation and bleedings, and genotyping has been seen as a potential means of preventing these events. However, the increased time within the therapeutic interval seen in the study by Caraco et al was not mainly due to a reduced frequency of too high INR values[263]. Rather, genotyping eliminated a large proportion of the too low values. The reason for this is that genotyping allowed the administration of doses significantly higher than the standard ones in individuals with the high-dose CYP2C9*1/*1 genotype.

To avoid bleedings in individuals genetically predisposed for a low dose requirement, standard warfarin induction involves low doses and a slow dose escalation. The price paid for this is that patients with a high dose requirement initially receive too low doses and risk prolonged under-anticoagulation. Although this should not cause thrombosis, because of the concomitant administration of heparins, extended periods of under-
dosing increase the number of visits for INR measurements and parenteral administration of heparins. Although this may seem trivial compared to the risk of bleedings in warfarin-sensitive patients, the under-dosing affects a much larger proportion of the patients. Therefore, the opportunity to safely increase the dose in patients lacking variant CYP2C9 or VKORC1 haplotype A alleles may very well turn out to be an economically important aspect of applied warfarin pharmacogenetics.

None of the prospective studies performed so far have extended the use of pharmacogenetics beyond the initial prediction of a suitable maintenance dose. It is yet unknown whether more sophisticated strategies with individualised follow-up and dose refinements based on both INR measurements and genotype information could increase the benefits of genotyping.

**4. Is genotyping cost-effective?**

Even if we conclude from the few clinical trials available that genotyping could improve the outcome of warfarin treatment, it is still difficult to determine its cost-effectiveness. On the cost side we have the expenses associated with the genotyping and possibly a delayed start of warfarin therapy (if genotyping results cannot be obtained immediately). A recent pharmacoeconomic study estimated a cost of genotyping amounting to $400[264], but the price is likely to fall if genotyping becomes a routine analysis. On the beneficial side we have a potential reduction in the number of bleedings and thromboses. In addition, the number of INR measurements could probably be reduced if the maintenance dose is reached faster and the INR level is more stable over time. Unfortunately, available data do not allow an estimation of the genotyping’s effect on these outcomes. However, observational studies could give us rough estimates of the number of adverse outcomes attributable to specific genetic factors. For example, Higashi et al demonstrated a 2.39-fold increased risk of major bleeding in patients with variant CYP2C9 alleles (31.4% of the patients), compared to homozygous CYP2C9*1 carriers[161]. These figures allow us to calculate the fraction of major bleedings that is attributable to variant CYP2C9 alleles, 30% *. Under the assumption that pharmacogenetically tailored warfarin therapy will not improve outcome in patients with the wild-type genotype, this figure indicates that genotyping of CYP2C9 could at best eliminate 30% of major bleedings in warfarin-treated patients. In other studies[167, 168, 170], 28-54% of the major bleedings have been attributed to variant CYP2C9 alleles. Although this has not been shown, some of the remaining bleedings may be attributable to VKORC1 polymorphisms. Consequently, genotyping of CYP2C9 and VKORC1 has a theoretical potential of preventing a sizable proportion of all major warfarin-associated bleedings. A meta-analysis of the three published prospective studies indicated that the risk of major bleeding during the first month of warfarin treatment was 32% lower in patients subjected to pharmacogenetic dosing, but the difference was non-significant and therefore compatible with zero effect[264].

*{(events in variant carriers) - (events in variant carriers had they been *1/*1 carriers)}/[total number of events] or 
(0.314*2.39-0.314*1)/(0.314*2.39+0.686)
The data provided in Higashi’s study also allows a further characterisation of the predictive properties of CYP2C9 genotyping. If for example the presence of at least one variant CYP2C9 allele is seen as a risk indicator of severe or life-threatening bleeding, the genotyping test has a sensitivity (the proportion of patients with bleeding that are correctly identified) of 50% and a specificity (the proportion of patients without bleeding that are correctly identified) of 73%. The modest performance of the test reflects the fact that many bleedings occur for reasons other than the presence of a variant CYP2C9 allele, and that most individuals with such high-risk alleles still do not experience a bleeding. The positive predictive value of the test (the proportion of patients identified as high-risk individuals that actually experience a bleeding) is 28%, while the negative predictive value is 87% reflecting the relative scarcity of bleeding events. However, to look at the genotyping as a binary test aiming at dividing the patients into a low-risk and a high-risk may not be entirely feasible, since the proposed use of the genetic information is to individualise the therapy in each patient.

In the meta-analysis of randomised studies mentioned above, 3.3% of the patients that were not genotyped experienced a major bleeding during the initiation of warfarin treatment[264]. The genotyping strategy reduced this number to 1.9%. These figures can be utilised to calculate the number needed to test, i.e. the number patients that would have to be genotyped in order to prevent one major bleeding. Provided that the estimates are correct (a somewhat brave assumption considering the non-significant result of the meta-analysis), the number needed to test is 73. If the additional cost associated with genotyping is $400, the cost of avoiding one major bleeding during warfarin induction would be 73 times $400, or $29200. Any effects of genotyping extending beyond the first 1-3 months are not included in this estimate, and it is only applicable to patient populations resembling those included in the meta-analysis. For example, only 0.6% of the patients included in the WARG cohort experienced a major bleeding (WHO definition) during the first three month of treatment (paper II), resulting in a number needed to test of approximately 400.

When discussing the cost-effectiveness of pharmacogenetic warfarin dosing, one should also acknowledge that a strict demand for cost-effectiveness is in itself disputable. Many of the diagnostic laboratory tests routinely used in clinical medicine today are probably not cost-effective, at least not when used non-discriminately, as is often the case[276, 277]. In addition, the cost-effectiveness requirements are usually less strict for non-dispensable therapies in severely ill patients[278]. Consequently, the demands for cost-effectiveness are not evenly distributed in medicine and it is not self-evident why the prevention of avoidable debilitating or even lethal side-effects in predisposed patients should be considered a procedure where cost-effectiveness is non-negotiable.

To summarise, it is yet uncertain whether genotyping should be routinely performed in warfarin-treated patients or not, although the concept is promising. Hopefully, large ongoing randomised trials will tell us whether pharmacogenetics is actually a powerful means of preventing severe side-effects of warfarin, or merely a dead-end research field.
5 ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to all those who have contributed to this thesis, and in particular:

My main supervisor professor Anders Rane and co-supervisor professor Marja-Liisa Dahl for excellent guidance and support.

Lennart Holm whose enthusiastic and dedicated work was essential to the project.

Marine Andersson, Mia Wadelius, Niclas Eriksson, Lars Alfredsson and the late Stefan Lundgren for rewarding co-authorship.

Birgitta Ask for her valuable help with DNA extraction and genotyping.

Margit Ekström for guiding me through the labyrinths of KI formalities.

Carl-Eric Elwin for introducing me to Clinical Pharmacology back in the summer of ’95.

Filip Josephson, Sweden’s foremost expert, for a good friendship and endless excursions into statistics, pharmacokinetics, and the nature of man.

Erik Eliasson and Leif Bertilsson, for helping me getting a grip on the cytochrome P450s, among other things.

Lars Ståhle for introducing me to the wonderful world of pharmacokinetics.

Ayman Al-Shurbaji for guiding me through my first research project.


My father-in-law Anders Sundqvist for restlessly inquiring me about the progress of the thesis.

My sister Josefin Silverfur for making me used to opposition from an early age and my brother-in-law Rikard Silverfur for being toastmaster on all important occasions in my life.

My parents Göran and Agneta for making me believe that everything is possible.

My wife Åsa and my daughters Maria and Erika for everything.

This research project was supported by the Swedish Foundation for Strategic Research, the Swedish Science Council (Medicine 04496), Nycomed AB, and the Swedish Drugs and Therapeutic Committee of Southwest Stockholm.
6 REFERENCES

5. Link, K.P. *Warfarin rodenticide bait composition and process of making same.* 2687365 (Patent) 1954
12. Bergman, U. Dept. of Clinical Pharmacology, Karolinska University Hospital. Personal communication. 4th September 2008


221. Osman, A., C. Enstrom, K. Arbring, P. Soderkvist, and T.L. Lindahl, Main haplotypes and mutational analysis of vitamin K epoxide reductase (VKORC1)


