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**Studies on warfarin treatment with  
emphasis on inter-individual variations  
and drug monitoring**

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Dedicated to my late mother,  
Abyan Yusuf



# ABSTRACT

Warfarin was introduced more than 60 years ago and is used worldwide for the prophylaxis of arterial and venous thromboembolism in primary and secondary prevention. The drug is orally administered as a racemic mixture of (R)- and (S)-enantiomers. The (S)-form is mainly responsible for the anticoagulant effect and is metabolised by *CYP2C9* enzyme in the liver microsomes. Warfarin exerts its pharmacological action by inhibiting the key enzyme (*VKORC1*) that regenerates vitamin K from an oxidised state to a reduced form. The latter is a cofactor for the post-translational modification of a number of proteins including coagulation factors II, VII, IX and X. The vitamin K-dependent modification provides these factors with the calcium-binding ability they require for the interaction with cell membranes of their target cells such as platelets.

Warfarin is monitored by measuring prothrombin time (PT) expressed as INR. Two main methods exist for PT analysis. The Owren method is used mainly in the Nordic and Baltic countries, in Japan, whereas the Quick method is employed in most other countries. Warfarin management is associated with some complications. Unlike many other drugs the dose for a given patient cannot be estimated beforehand, dose-response relationship is not predictable, and the prevention of thrombosis must be balanced against the risk of bleeding. Furthermore, the different PT methods used to monitor the drug are sometimes not in agreement and show significant discrepancies in results.

In an attempt to clarify the mechanisms influencing the inter-individual variations in warfarin therapy and to detect the factors that contribute to differences between PT methods, studies were conducted in collaboration with hospitals and anticoagulation clinics in the south-eastern region of Sweden. First, a stereo-specific HPLC method for measurement of warfarin enantiomers was developed and validated. With this method, the levels of plasma warfarin following its oral administration can be studied and evaluated. Abnormal clearance in some patients can be detected, and patient compliance can be verified. Furthermore, differing ratios of (S)- and (R)-isomers can be identified.

The impact of common *VKORC1* polymorphisms on warfarin therapy was investigated. This study has shown that the *VKORC1*\*2 haplotype is an important genetic determinant for warfarin dosage and is associated with difficulties in attaining and retaining therapeutic PT-INR. Further, significant differences in warfarin S/R-ratio was detected between patients with *VKORC1*\*2 and *VKORC1*\*3 or *VKORC1*\*4 variants. This difference was not coupled with *CYP2C9* genotype.

The effects of predilution of patient plasma samples, sources of thromboplastin and deficient plasma on between PT methods agreement were studied. This study has revealed that sample predilution according to the Owren method is to be preferred for the harmonisation of PT results. Undiluted samples, in contrast, according to the Quick method have shown reduced correlation between two different thromboplastin reagents. Sources of thromboplastin and deficient plasma were only of minor importance.

# LIST OF THE PAPERS

This thesis is based on the work presented in the following Papers:

- I. Osman A, Arbring K, Lindahl TL. A new high-performance liquid chromatographic method for determination of warfarin enantiomers. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005;**826**:75-80.
- II. Osman A, Enstrom C, Arbring K, Soderkvist P, Lindahl TL. Main haplotypes and mutational analysis of vitamin K epoxide reductase (VKORC1) in a Swedish population: a retrospective analysis of case records. *J Thromb Haemost* 2006;**4**:1723-1729.
- III. Osman A, Enström C, Lindahl TL. Plasma S/R ratio of warfarin co-varies with *VKORC1* haplotype. *Blood Coagul Fibrinolysis*. In press
- IV. Osman A, Lindahl TL. Plasma sample dilution improves the correlation between reagents for PT methods. Submitted

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# ABBREVIATIONS

CV	Variation Coefficient or coefficient of variation
CYP	Cytochrome P 450
ddNTP	Dideoxynucleotide triphosphate
DHPLC	Denaturing HPLC
dNTP	Dinucleotide triphosphate
ER	Endoplasmatic reticulum
GGCX	Gamma-glutamyl carboxylase
Gla	Gamma-carboxyl glutamic acid
Glu	Glutamic acid
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
ICC	International calibration constant
i.e.	Id est (that is to say; in other words)
INR	International normalised ratio
IRP	International reference preparation
IS	Internal standard
ISI	International sensitivity index
e.g.	Exempli gratia (for example)
et al.	Et alii (and others)
KH <sub>2</sub>	Vitamin K quinol
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
NF1	Nuclear factor 1
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
PDA	Photo diode array
PIVKA	Protein induced by vitamin K absence or antagonist
PT	Prothrombin time
QC	Quality control
SD	Standard deviation
SNP	Single nucleotide polymorphism
ssDNA	Single-stranded DNA
UV	Ultra violet
VKA	Vitamin K antagonist
VKO	Vitamin K oxide (vitamin K epoxide)
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1
VKR	Vitamin K reductase
WHO	World health organisation



# INTRODUCTION

## Historical review

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"One sometimes finds what one is not looking for."

Alexander Fleming

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### ***The discovery of warfarin***

The inter-war period of the 1920s and 1930s was a remarkable time, characterised by many uncertainties, fear and economic depression, but also creativity, scientific progress and inventions. In Europe, surrealism became a movement that influenced art and philosophy. André Breton published the *Surrealist Manifesto* in 1924, in which he considered dreams as a key to the subconscious. In science, the relativity theory of Einstein was followed by the quantum mechanics of Bohr. Spin physics was introduced, and Heisenberg shocked the world with his *uncertainty* principle. No one knew any longer where the electrons were hiding in the atoms. Not even Einstein had any idea about that. Sceptical as he was about the new quantum physics, Einstein wrote in 1926: "*The theory yields a lot, but it hardly brings us any closer to the secret of the Old One. In any case I am convinced that He does not throw dice.*"

It was also an era when mould cropped up where it was least expected. The British bacteriologist, Alexander Fleming, one day in 1928 cleared up his cluttered laboratory and found mould on a glass plate that he had previously coated with staphylococcus bacteria. His observation led to the discovery of penicillin, one of the most important drugs ever invented in human history.

Mould was also behind the discovery of warfarin and other coumarin derivatives, though in tragic circumstances. In the early 1920s, farmers in North America reported a new cattle disease that was characterised by fatal bleeding. A cattleman dehorned 80 calves and most of them died of spontaneous haemorrhage within hours. Of 25 castrated young bulls 12 died of internal bleeding. Veterinarians who investigated the animals concluded soon that the disease was not caused by infectious microbes or by a nutritional deficiency. Frank Schofield, a veterinary pathologist in Canada, eventually recognised that the disease originated from the consumption of mouldy hay made from sweet clover (*Melilotus officinalis*) [1]. Then, in 1929 Roderick, a veterinarian in the USA, demonstrated that the spoiled sweet clover contained an unknown anticoagulant agent interrupting the function of prothrombin [2]. Roderick carried out a series of studies on the pathophysiology of the disease and suggested that the sweet clover disease involved a reduction of prothrombin and consequently a prolongation of coagulation time [2].

Roderick and Schofield suggested that the disease was reversible. The outbreak could be controlled by removing the contaminated hay from the diet and by blood transfusions from healthy animals [1,2]. Roderick's finding was later confirmed by Armand J. Quick, a world authority on blood coagulation and prothrombin function [3]. Quick also acknowledged that

alfalfa, a plant rich in vitamin K, provided a curative effect against the sweet clover disease, although he wrongly denied that vitamin K itself could function as an antidote for the disease. The newly discovered vitamin K [4,5] was concurrently attracting an increasing interest, parallel to the agent in the sweet clover disease and would later be shown to be a remedy for the bleeding disorder induced by the substance in the spoiled hay.

In 1933, at the Biochemistry Department of University of Wisconsin, USA, Karl P. Link and his co-workers received a milk can full of blood that had lost clotting ability and 45 kg of spoiled sweet clover from a desperate farmer whose cattle were infected with the sweet clover disease [6]. The farmer had lost many of his animals and, in a time of economic depression, needed urgent help. But Link could not offer him that immediate help and could only repeat the recommendations of Schofield and Roderick to “stop feeding that hay” [6]. Link and his co-workers, nevertheless, took the samples, and based on the pioneering work of Roderick, they started an extensive research to isolate and characterise the toxic substance in the sweet clover. They used a modified version of the newly established 1-stage method of Quick [7] to measure the clotting time. The agent in the spoiled sweet clover was given the code name H. A. (haemorrhagic agent). In June 1939, Campbell, a co-worker of Link’s, finally succeeded in isolating a crystalline of agent H. A. [8,9]. The new substance was named 3,3'-methylenebis(4-hydroxycoumarin) or dicumarol and was, as previously predicted, an inhibitor of prothrombin function. Shortly thereafter, Link’s group synthesized dicumarol and conducted dose-response trials on rabbits, rats, guinea pigs, mice and dogs [6,10]. They also suggested that the observed hypoprothrombinaemia caused by dicumarol could be induced by some of its analogues and derivatives, and that vitamin K reversed the depleted prothrombin [6,10]. In 1940, the first patients were treated with dicumarol as a prophylaxis against thromboembolic diseases [6]. Within 2 years after the synthesis of dicumarol, over 100 analogues of 3-substituted 4- hydroxycoumarins were prepared in Link’s laboratory. One of them, code number 42, was highly potent and very effective in rodent control [6]. Link and his co-workers named the new substance WARFARIN, by combining the first letters of the Wisconsin Alumni Research Foundation (who sponsored the project) with ARIN from coumarin. Warfarin, in contrast to dicumarol, contained an asymmetric carbon, giving it a possibility for chirality (Figure 1).

Despite its recognised effect as “blood thinner”, most clinicians and scientific society were initially sceptical to the new drug. Many doubted that a “cow poison” and a product intended for rodent control would ever be useful in human therapy. Others opposed the idea that vitamin K could function as an antidote to coumarin poisoning. But Jörgen Lehmann (in Sweden) and others subsequently showed that vitamin K could neutralise the anticoagulant action of coumarins (in [6]). Lehman, who is recognised for his discovery of the anti-tuberculosis drug PAS (para-amino salicylic acid), had been a student under Torsten Thunberg, the discoverer of dehydrogenases, at the Sahlgrenska hospital in Gothenburg and was well-familiar with substrate inhibitions of enzyme activity. Lehmann emphasised the structural similarities between coumarin and the naphthoquinone part of vitamin K. He was the first to realise that coumarins interrupt the process in which vitamin K participates as a substrate for the formation of functioning prothrombin. The war, however, had delayed the publication of some of his findings in international journals, as Lehmann revealed in his historical article in *Circulation* from 1959 [11].

By the early 1950s, sodium warfarin was introduced into the market as human oral anticoagulant by Endo-Laboratories (Richmond Hill, N.Y., USA) under the trade name Coumadin Sodium [6]. Perhaps one of the most famous early patients was President Eisenhower of the USA., who in 1955 was treated with warfarin after developing coronary

thrombosis [12]. In Sweden, dicumarol was introduced in the early 1940s, where Lehmann in 1942 published the first clinical reports from Gothenburg [11].

Almost 60 years after its discovery, warfarin remains the world's most frequently used drug for the treatment and prevention of thromboembolic events. One of its early recognised advantages was that it could be taken *per os* rather than parenterally, as is the case with heparin, another anticoagulant discovered before coumarins [13]. The history of heparin and aspirin and their discovery as drugs for thrombotic diseases is also fascinating but is reviewed elsewhere [14].

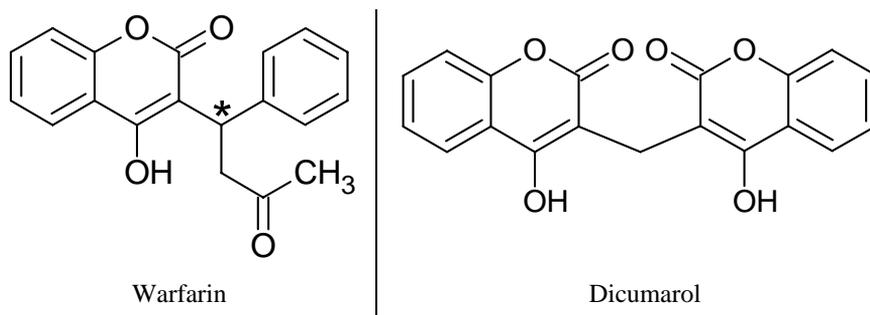


Fig. 1. Molecular structures of warfarin and dicumarol. Asterisk indicates chiral centre.

### **The discovery of vitamin K**

Parallel with the research on the sweet clover disease in the North America, Henrik Dam was working in Copenhagen, Denmark, with sterol metabolism. During 1929 – 1930, he observed that chicks fed an artificially prepared diet with minimal amounts of cholesterol had a marked tendency to subcutaneous and intramuscular bleeding as well as some abdominal pathologic changes [4]. The symptoms could not be prevented by addition of cholesterol or cod-liver oil. The condition resembled scurvy (*Scorbutus*), a disease that results from vitamin C deficiency. However, adding extra vitamin C to the diet had no effect on the disease, and Dam concluded that the lack of an unknown factor or factors was the cause of the observed symptoms [4].

Dam knew that the factor was fat-soluble, but not identical to vitamin A, D or E. He carried out a series of chemical extractions on different food sources, testing cereals and seeds, vegetables, animal organs, different fats and oils, and hen's egg [5]. After a number of fractionations, he found high activities of the unknown factor in hog-liver fat, whereas cod-liver oil contained only very limited amounts. Hemp seed (*Cannabis sativa*) was also a good source. Dam gave the term *Vitamin K* to the new essential factor, with “K” referring to the word *koagulation* in the Scandinavian and German languages [5].

In the following years, extensive research, particularly by the co-workers of Doisy [15-17] and Fieser [18,19], led to the isolation and synthesis of the vitamin. Doisy's co-workers were the first to isolate vitamin K<sub>1</sub> (phylloquinone) and vitamin K<sub>2</sub> (menaquinones) and they also

predicted the molecular structures of these two related vitamin K molecules [15,17], both containing a functional naphthoquinone ring and an aliphatic side chain (Figure 2). In 1939, Fieser synthesised vitamin K<sub>1</sub> and confirmed the structure predicted by Doisy and co-workers [18,19]. A year later, Fieser's group synthesised a number of vitamin K<sub>1</sub> derivatives including vitamin K<sub>1</sub> epoxide [20]. The epoxide is still synthesised by this method.

A therapeutic application of vitamin K came soon when Dam and co-workers demonstrated that the hypoprothrombinaemia syndrome observed in newborn babies could be cured by injecting vitamin K (in [21]). Prophylaxis with vitamin K for all newborn infants is currently exercised in many countries and has been shown to be an effective way of preventing intracranial haemorrhages caused by vitamin k deficiency [22]. For his work on vitamin K, Dam was awarded the 1943 Nobel Prize in medicine, which he shared with Doisy.

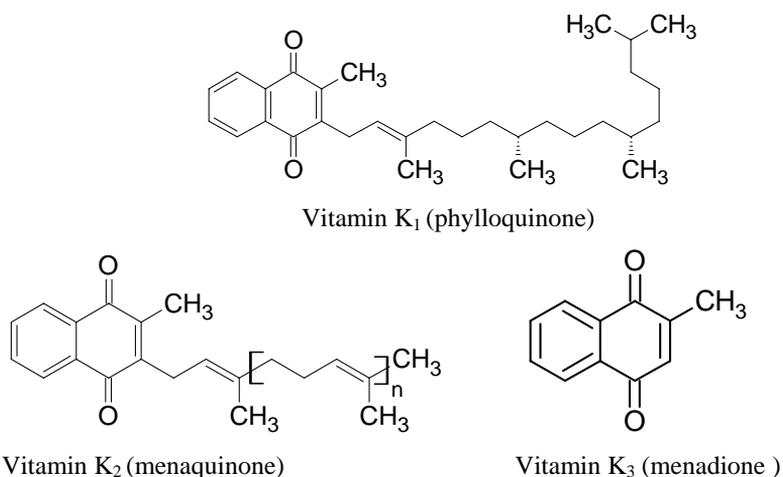


Fig. 2. Molecular structures of vitamin K<sub>1</sub>, K<sub>2</sub> and K<sub>3</sub>. The letter "n" denotes a variable number of isoprenoid units.

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## **Warfarin therapy: *clinical indications and adverse events***

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Warfarin and other coumarin derivatives are collectively often termed as coumarins, anti-vitamin K drugs or vitamin K antagonists (VKA). They are useful for the prophylaxis of arterial and venous thromboembolism, in primary as well as secondary prevention. VKA are applied as a preventive measure against systemic embolism in patients with prosthetic heart valves or atrial fibrillation, for the primary prevention of acute myocardial infarction and for the prevention of stroke and recurrent infarction in patients with acute myocardial infarction [23]. Both early and later randomised clinical trials have also shown that oral anticoagulants are effective in the prevention of venous thrombosis after hip surgery [12]. The extent and the type of thrombus often influence the course of prophylaxis. Longer duration of therapy, for instance, is needed for patients with proximal deep venous thrombosis or recurrent venous thrombosis than those with a single episode [24]. In Sweden, Schulman and others [25] performed in 1995 a randomised, multi-centre trial comparing 6 weeks and 6 months of anticoagulation therapy with warfarin or dicumarol. Their result showed that 6 months of coumarin oral anticoagulation following a first episode of venous thromboembolism gave a lower recurrence rate than treatment with a 6-week period. That study was later confirmed by Kearon et al. [26], who carried out a double-blind, multi-centre, randomised trial in which patients with venous thromboembolism were assigned to 3 months or 2 years of warfarin treatment.

A lag period of 4 – 6 days is usually required before the antithrombotic effect of VKA is observed, although the anticoagulant effect of the drug appears already after two days. The anticoagulant effect refers to the prolongation of coagulation time, which is observed 36 – 48 hours after the first dose of the drug. On the other hand, the clinically more important antithrombotic effect is reflected by the prevention of clots and is believed to largely depend on the clearance of prothrombin, which has a relatively long half-life of up to 5 days [24]. It is therefore common that patients with different thromboembolic events start 5 – 10-day courses of heparin before a long-term VKA therapy is set [27]. A large number of randomised clinical trials have been performed in the past to evaluate the benefits of warfarin therapy in different dose intensities, in different durations, in different cardiovascular conditions, and in different patient populations. These studies are summarised in review articles by Hirsh, Ansell and others [12,23,24,28,29].

Although the introduction of warfarin and other coumarin derivatives has been of huge benefit for patient care and has saved many lives, several complications are associated with the use of these VKA drugs. Considerable inter-individual dose variations and adverse events including bleeding risk were already reported in the 1950s [30,31]. Dose variations can be explained by a number of different factors including genes, nutritional status, age, and concomitant drug intake. Bleeding risk remains a constant threat to patients and concern for doctors [24]. The intensity of anticoagulant therapy is regarded to be an important factor influencing the risk of bleeding, with elderly patients being a high-risk group [29]. Two types of adverse events are usually classified: minor and major bleeding. Minor haemorrhage is normally reported but needs no additional main investigations or interventions. Major bleeding risk, including fatal or life-threatening episodes, has been estimated at 1 – 3% per year [32] and often leads to urgent hospitalisation [24]. Recent studies indicate that daily and simultaneous supplementation with vitamin K in unstable patients may prevent excessive anticoagulation and risk of bleeding without raising the risk of thromboembolic events [33,34]. Adverse events and the number of visits that patients need to make to the health care

system are also an economic burden on society. Anticoagulated patients often need to be tested either in anticoagulation clinics or in the usual care to monitor their drug response.

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## Stereochemistry

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Nature gives many examples of stereoisomerism. The two human hands are each other's mirror image but not identical. It is difficult to fit the right foot in a left foot's shoe. Stereochemistry describes how the atoms in molecules are arranged in three-dimensional space. An important component of stereochemistry is molecular symmetry, defined mathematically by *group theory* [35]. Common symmetry elements include: symmetry plane ( $\sigma$ ), inversion centre (i), proper rotation axis ( $C_n$ ) and improper axis ( $S_n$ ). The most important symmetry element for organic molecules is  $\sigma$ . However, the vast majority of the organic molecules occurring in nature, including numerous indigenous substances, nutrients and many drugs, have no symmetry. A molecule lacking a symmetry plane is not identical to its mirror image and is said to be *chiral* (from the Greek *cheir*, "hand"). In contrast, molecules with a plane of symmetry contain two identical halves and are called *achiral*.

The most common cause of chirality in organic molecules is the presence of a tetrahedral,  $sp^3$ -hybridised, carbon atom bonded to four different groups (Figure 3). The tetrahedral configuration of carbon is described by the VSEPR theory (valence-shell electron-pair repulsion), which predicts that the valence electron pairs of any structure will prefer to be as apart as possible and repel each other. The bond configuration of a tetrahedral carbon will thus adapt a geometry that makes the angles of all bonds  $109.5^\circ$ . If at least two of the substitutes of the tetrahedral carbon are identical, a  $\sigma$ -symmetry will exist, and the molecule is identical to its mirror image. On the other hand, a tetrahedral carbon bonded to four different groups is chiral and exists as a pair of non-superimposable, mirror-image isomers called enantiomers (Greek *enantio*, "opposite") (Figure 3). A mixture of such a pair of enantiomers is called a racemic mixture.

In CIP convention (Cahn-Ingold-Prelog), the configuration of enantiomers can be specified as either R (latin *rectus*, "right") or S (*sinister*, "left") [36]. The CIP convention uses a system called *sequence rules*, where the four substitutes of a chiral carbon are assigned priorities. Depending on these priorities, the orientation of the molecule is either R (clockwise) or S (counter-clockwise). The chiral carbon of warfarin (C9) gives rise to two enantiomers; (R)- and (S)-warfarin (Figure 4) [37]. These two have similar physical properties except for their direction in which they rotate plane-polarised light. In the biological systems, however, (R)- and (S)-warfarin enantiomers have distinct pharmacokinetics and are metabolised by different enzymes, although both isomers exert their anticoagulant effect by inhibiting the same target receptor, vitamin K epoxide reductase (VKOR) [38].

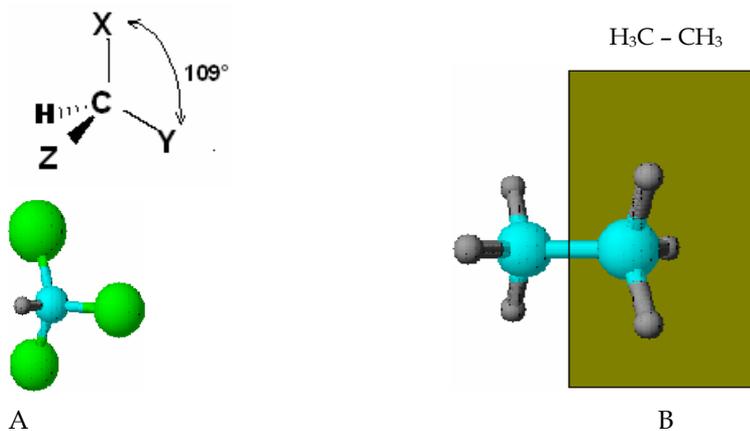


Fig. 3. A tetrahedral structure of a carbon atom bonded to four different groups (A). All bond angles are  $109^\circ$ . Such structure has no plane of symmetry and is said to be chiral. In contrast, ethane contains a plane of symmetry (B), is not chiral and is identical to its mirror image.

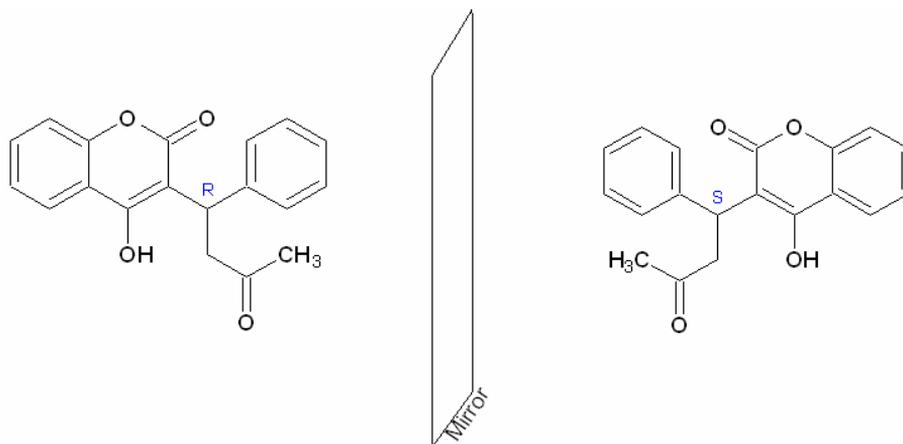


Fig. 4. Warfarin (R)- and (S)-enantiomers. The two molecules are each other's mirror image.

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## Pharmacokinetics

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Warfarin is rapidly absorbed from the gastrointestinal tract, with almost 100% bioavailability and similar distribution volumes for the two enantiomers [38]. The drug binds extensively to plasma proteins, principally to human serum albumin (HSA) [39], and it is only the unbound fraction of warfarin that has a pharmacological activity [38]. On the HSA protein, several sites are available for ligand interaction and binding [40]. Warfarin binds to an area known as the azapropazone binding site [41]. Previous studies have proposed a two-step binding model, where the first step is rapid and the second is facilitated by a conformational change of the protein to form a stable HSA-warfarin complex [42]. The (R)-isomer has been observed to bind to HSA with higher affinity than the (S)-enantiomer [42]. The two enantiomers also differ in their clearance and metabolic pathways. At steady state, the plasma level of (R)-warfarin is 1.5 times higher than that of (S)-enantiomer, as we have demonstrated in 141 stable anticoagulated patients [43]. The plasma half-times of (S)- and (R)-warfarin have been estimated at 29 and 45 hours, respectively [38].

Warfarin undergoes stereoselective and regioselective metabolism in the liver by cytochrome P450 (*CYP*) enzymes [44-46]. Stereoselective metabolism is defined by the existence of a carbon chiral centre, which in warfarin is at position 9. Thus, (R)- and (S)-isomers are stereoselectively distinguished by their metabolising enzymes. Regioselectivity, on the other hand, refers to the sites on the molecule that are hydroxylated. The oxidative phase I biotransformations of warfarin were studied by Trager et al., who identified several monohydroxylated metabolites including 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin (Table 1) [47]. Furthermore, cis- and trans-dehydrowarfarin, and two diastereomeric alcohols, have been found as metabolic products. These hydroxylations have been shown to be the activity of different *CYP* isoenzymes [44]. The principle enzyme metabolising (S)-warfarin was initially termed as high affinity (S)-warfarin 7-hydroxylase, but has been identified as *CYP2C9* (cytochrome P450, family 2, subfamily C, polypeptide 9) [48]. This enzyme is regioselective mainly for carbon 7 of (S)-warfarin, yielding 7-hydroxywarfarin, and to a lesser extent for 6-hydroxywarfarin [45]. The metabolism of (R)-warfarin is, however, more complex and is still a matter of debate. *CYP1A2* has been suggested to be stereoselective for the formation of 6-, 7-, and 8-hydroxy (R)-warfarin, with regioselectivity for the 6-position [49]. But it is believed that *CYP3A4*, *CYP2C8*, *CYP2C18*, and *CYP2C19* are also involved in the bioconversions of (R)-warfarin [45].

Warfarin is further processed in phase II metabolism. In rats, glucuronidation appears to be the predominant pathway for 4'-hydroxywarfarin, whereas 6-hydroxywarfarin is found mainly as sulphate conjugates [50]. In humans, phase II metabolism of warfarin remains unclarified [51]. It is nevertheless believed that the drug is fully metabolised, as no significant amounts of unchanged warfarin have been found in the urine of healthy subjects [51].

Table 1. The main hydroxylation products formed in phase 1 metabolism of warfarin and the enzymes involved in their biotransformations [38,51].

Enantiomer	Enzyme	Main metabolites	Remarks
(S)-warfarin	<i>CYP2C9</i>	7-hydroxywarfarin	Main product
		6-hydroxywarfarin	Minor product
(R)-warfarin	<i>CYP1A2</i> <i>CYP3A4</i> <i>CYP2C8</i> <i>CYP2C18</i> <i>CYP2C19</i>	4', 6-, 7-, 8, and 10-hydroxywarfarin	<i>CYP1A2</i> is regarded to be the principle enzyme for the metabolism of (R)-warfarin and is regioselective primarily for 6-OH warfarin [38,49].

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## Pharmacodynamics

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Warfarin and other VKA drugs exert their anticoagulant effect by inhibiting the recycling of vitamin K [52]. In haemostasis, factors II (prothrombin), VII, IX, and X require vitamin K for their biological activity [53]. The anticoagulant proteins C and S are also vitamin K-dependents. Thus, warfarin exercises a concurrent suppression on both coagulation and anticoagulation pathways of haemostasis. The inhibition of vitamin K-dependent coagulation factors leads to decrease in thrombin generation. Consequently, the formation of fibrin – the final product of the clotting cascade – will decline (Figure 5).

Pharmacodynamically, (R)- and (S)-warfarin act on the same target site. However, the (S)-enantiomer has been reported to have 2 – 5 times more potency than its (R) analogue [54]. This stereospecific potency is believed to be the result of differences in affinity for the target receptor, VKOR, that is inhibited by warfarin [38].

The vitamin K-dependent factors have differing half-lives in plasma and hence are depressed at different rates during warfarin treatment. After initial oral anticoagulant administration, factor VII is the first to decrease due to its shorter half-life, whereas depression of prothrombin occurs slowly and takes several days [55]. Thus, the antithrombotic effect of the drug is delayed until the levels of factors II and X are reduced. The dose of administered warfarin is, however, important for the balance between coagulation and antithrombotic protection. If the initial dose is too large, the levels of protein C are rapidly depleted, leading to hypercoagulation [56].

The relationship between dose and response of warfarin is unpredictable. There is no significant correlations between clotting time and maintenance dose, or between clotting time and plasma concentrations of warfarin enantiomers [43,57]. Dose adjustment on an individual basis is, therefore, necessary in almost all cases.

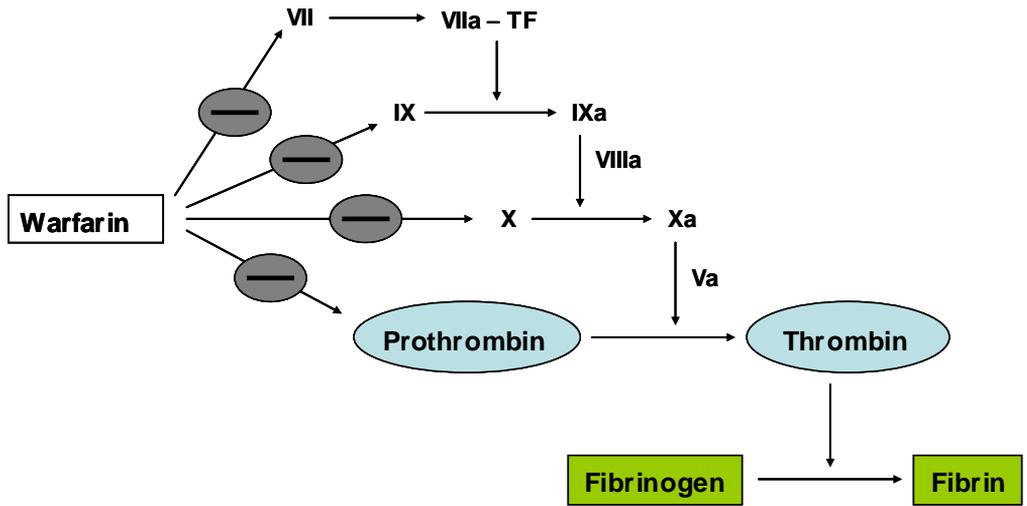


Fig. 5. The tissue factor (TF) pathway of haemostasis and the inhibitory action of warfarin on vitamin K-dependent coagulation factors.

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## Warfarin monitoring

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### ***The evolution of prothrombin time***

Management of warfarin and other VKA drugs is associated with some uncertainties. Initiation of the treatment is not straightforward in most cases, because the patient's initial dose is not known beforehand. Dose-response relationship is not predictable, and the prevention of thrombosis must be balanced against the risk of bleeding. Therefore, repeated analysis of patient's clotting time is required during the therapy.

The measurement of clotting time (coagulation time) was introduced in the 1930s by Armand Quick [7]. This occurred nearly a decade before VKA drugs were established as oral anticoagulants for human therapy. At that time, the classical coagulation theory of Morawitz was still universal (in [58]). The one-stage method of Quick was based on the then widely accepted four-factor theory postulating the following scheme [7].

Prothrombin + thromboplastin + calcium = thrombin

Fibrinogen + thrombin = fibrin

The four-factor model assumed that if calcium and thromboplastin, a tissue factor that initiates blood clotting, were present in sufficient amounts, the clotting time was determined by the only remaining variable, namely prothrombin. Fibrinogen was supposed not to disturb to an extent that prolonged clotting time. The Quick test was hence termed prothrombin time (PT) and is still used worldwide to monitor warfarin and other VKA drugs. However, the classical four-factor theory is no longer valid and has many times been revised as the repertoire of coagulation factors has grown.

Despite its name, PT does not measure the activity of prothrombin alone. PT is prolonged by the combined reductions of factors II, VII, and X, which are all vitamin K-dependent. Reduction of the fourth vitamin K-dependent coagulation factor, IX, does not prolong PT [53]. Furthermore, the Quick PT is dependent on the levels of factor V and fibrinogen, and involves a procedure where one part of undiluted plasma sample is mixed with two parts of a reagent containing equal parts of thromboplastin and calcium to give a final sample dilution of 1:3.

The original Quick test has been modified several times. In Norway, in 1942, Paul Owren examined a patient who was admitted to the university hospital in Oslo for a haemorrhagic disease [58]. The patient's PT was prolonged but the cause of the disease was not lack of prothrombin as the defect could be corrected by prothrombin-free plasma prepared by adsorption to  $Mg(OH)_2$ . The deficient clotting factor was called "unstable factor" but was later named factor V. It was soon followed by the discovery of factor VII, termed initially proconvertin (in [58]). Like prothrombin, this factor was found to be depressed by coumarin therapy. As a consequence of these observations, Owren reported the first modification of Quick's method in a report entitled "*The control of Dicumarol therapy and the quantitative determination of prothrombin and proconvertin*" (P & P test) [59]. The P & P test had several advantages over the original PT test of Quick [60]. The plasma was prediluted to increase the sensitivity and to minimise the effect of possible inhibitors originating from the sample collection tubes. Addition of adsorbed bovine plasma deficient of vitamin K-dependent factors but containing sufficient amounts of factor V and fibrinogen increased the specificity

of the test for those factors that were known to be affected by VKA treatment. The depleted plasma in the P & P test was prepared this time by passing the plasma through a filter containing asbestos [59]. The concentration of calcium ions was held constant and optimal amounts of thromboplastin were added.

A second generation of Owren assay called thrombotest was developed in 1959 [61] after the discovery of factor X (Stuart factor) [58]. This factor, like factors II, VII and IX, is affected by VKA therapy, and the new Owren test was now specific for all vitamin K-dependent coagulation factors. Thrombotest used an “all-in-one-reagent” containing the following components. 1) Crude cephalin (phospholipid) prepared by ether extraction of human brain or soya bean. 2) Thromboplastin prepared from animal brain. 3) Adsorbed bovine plasma depleted of vitamin K-dependent factors and adjusted to pH 7.3. 4) Calcium chloride in optimal concentrations.

A modified version of the original Owren methods is currently used predominantly in the Nordic and Baltic countries. This test is mainly based on Owren’s thrombotest but uses exclusively a thromboplastin extract from rabbit brain [62]. The sample is prediluted to 1:7 with buffer containing citrate before two parts of combined reagent containing depleted bovine plasma (produced by adsorption to barium salt) and thromboplastin are added. The final sample dilution in the clotting reaction is thus 1:21 compared with 1:3 in Quick methods [62]. The advantage of the current Owren method is that a minimal amount of sample is needed for the PT test, and the test is therefore suitable for laboratory analysis of paediatric and capillary blood samples. The dilution also reduces interference, which is desirable particularly when lupus anticoagulant is present [63].

### ***The International Normalized Ratio (INR) of PT***

For many years, the PT results were expressed in seconds, prothrombin index, prothrombin activity, or prothrombin ratio [53]. Hence, PT analysis was not standardised and the results from different laboratories or regions were not comparable. Furthermore, it became apparent that the different thromboplastins used in the PT reagents gave systematic variations on the results [64]. A thromboplastin reagent contains a tissue factor – a protein that initiates the clotting reactions via the extrinsic pathway of haemostasis (Figure 5). Brain and placenta are tissues rich in thromboplastin, and the protein is usually prepared from these organs. Recombinant thromboplastin has also been introduced and is now commercially available. Thromboplastins vary in their sensitivity to the PT test [53]. They may differ in the degree that they are inhibited by PIVKAs – the defective vitamin K-dependent factors that are present in plasma after inhibition by warfarin and other VKA drugs (in [65]). Furthermore, they vary in their dependence on factor V in the tested plasma, especially when Quick PT is used.

An increasing need to standardise PT measurements led to the introduction of the so-called Manchester Comparative Reagent in Britain, in the early 1960s [53]. The idea was that a single preparation of thromboplastin might be used as a reference material against which all other thromboplastins could be compared. Then, in 1973, the International Committee on Thrombosis and Haemostasis (ICTH) established five such preparations. A programme was established in which 199 laboratories participated in a PT standardisation trial supervised by an expert panel [66]. The result of this study led to the designation of a thromboplastin solution termed International Reference Preparation (IRP) by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organization (WHO) [65]. The IRP material, which was a combined Owren reagent containing human brain thromboplastin and

adsorbed bovine plasma, was given the code 67/40. The method of calibration was based on the assumption that if the PT ratios ( $PT_{\text{patient}}/PT_{\text{mean normal plasma}}$ ) for a number of patients obtained using two different thromboplastins are plotted against each other, a straight-line relationship is found [64]. The slope of this line was called the International Calibration Constant (ICC) and was assigned the value 1 for the IRP 67/40. This value was what it would have obtained if the IRP was calibrated against itself. From now, any thromboplastin would be weighed against the IRP, and PT results could be converted into corresponding values that would have been found if the IRP was used for the analysis.

Despite initial beliefs that the introduction of the IRP material would solve all problems, it soon became apparent that a worldwide PT calibration against the primary WHO IRP would face certain problems. The original IRP stock was limited and would be exhausted rapidly if every thromboplastin was calibrated against it [66]. Secondary IRPs were therefore designated, which were calibrated against the first 67/40 IRP. The secondary thromboplastins would then be used to calibrate other working preparations in place of the primary IRP. Several such intermediary preparations were established, which became obtainable from international organisations such as the Bureau Communautaire de Reference (BCR) of the European Community [53]. Thus, the calibration scheme became hierarchical with the primary 67/40 IRP on the top and all other thromboplastins in different positions on the chain [67].

Another problem facing PT calibration with IRP was that the assumption of a straight-line relationship between thromboplastin pairs was invalid in some cases. It was found that when thromboplastins of very different sensitivity were plotted against each other, the ICC of the straight line deviated markedly from the supposed value of 1 [64]. A new correction term called the International Sensitivity Index (ISI) was consequently introduced [66]. Instead of PT ratios, the PT itself (seconds) in the form of logarithmic values was used for the graph. The ISI is the slope of the calibration line obtained when  $\log PT$  for the IRP thromboplastin is set on the vertical axis and  $\log PT$  for a local thromboplastin is set on the horizontal axis [67]. From the ISI constant, the International Normalized Ratio (INR) is calculated according to the following equation [53].

$$\text{INR} = \text{Observed prothrombin ratio (PR)}^{\text{ISI}},$$

Where PR = patient clotting time/mean normal clotting time

The use of the INR has made it possible to carry out direct comparisons of PT results between different laboratories, regardless of the reagent used [67]. But PT calibration according to IRP calibration has some disadvantages in terms of cost and stability for the reference reagent due to transportation, storage and administration [68]. In the Scandinavian countries, where Owren's PT is used, an alternative calibration procedure was introduced in 1999 [68]. The proposed method utilised diluted normal plasma instead of IRP, and the percent of normal activity (PT%) was converted to the INR by using an equation derived from a regression analysis. In Sweden at present, the INR is calibrated using this method, and a reference thromboplastin (IRP) is therefore not required. A three-year follow-up in Sweden, in which several laboratories had participated in an external quality control programme, reported that the intra- and inter-laboratory variations had become markedly improved with this local INR calibration [62].

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## The vitamin K cycle and mechanism of warfarin action

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### **Vitamin K cycle**

Vitamin K is a family of structurally related fat-soluble compounds including phyloquinone ( $K_1$ ), menaquinone ( $K_2$ ) and menadione ( $K_3$ ). Rules of nomenclature for vitamin K molecules and other vitamins were described in 1967 by IUPAC-IUB Commission on Biochemical Nomenclature [69]. However, terms  $K_1$ ,  $K_2$ , and  $K_3$  have remained in use since their invention. All vitamin K molecules contain 2-methyl-1,4-naphthoquinone and are characterised by a naphthalene ring containing two carbonyl moieties at positions 1 and 4 (Figure 2). The best known of them is vitamin  $K_1$  (phyloquinone), which is found mainly in green vegetables. In chloroplasts, phyloquinone is an important molecule for energy transference in photosynthesis. Vitamin  $K_2$  (menaquinone) is synthesised by bacteria but is also found in liver, milk, cheese and fermented soy products [70]. Menadione ( $K_3$ ) is chemically synthesised as provitamin because vertebrate intestinal bacteria can convert it to  $K_2$  by adding a 4-prenyl side-chain at the 3-position.

Both  $K_1$  and  $K_2$  act similarly in haemostasis, but  $K_2$  is also involved in the promotion of bone development and has recently been shown to act as mRNA transcriptional factor in the regulation of bone-specific genes [70]. In haemostasis, procoagulant factors II, VII, IX, X as well as anticoagulant proteins C and S need vitamin K for their physiological function [71,72]. Furthermore, bone proteins osteocalcin and matrix Gla protein, protein Z, The AxI receptor ligand GAS 6, and four putative membrane proteins of unknown function, have been found to be vitamin K-dependent [33,73,74].

Although the anti-haemorrhagic activity of vitamin K epoxide was observed just a few years after the discovery of vitamin K [20], the role of vitamin K in haemostasis remained obscure until the 1970's. In the early 1960s, it was known that coagulation factors II (prothrombin), VII, IX, and X required vitamin K for their biological function. Hill et al. [75] recognised in 1968 that the function of vitamin K was not at genetic level, but rather at a later post-translation stage. In 1970, Bell et al. [76] described an enzymatic activity that regenerated vitamin K from vitamin K epoxide. An important milestone was reached in 1974 when Stenflo et al. in Malmö, Sweden, delivered an explanation for the role of vitamin K in haemostasis [72,77]. By using NMR spectroscopy and mass spectrometry, they isolated a tetrapeptide from the N-terminal of prothrombin. This peptide contained glutamic acid residues (Glu) that were modified to  $\gamma$ -carboxyl glutamic acid (Gla). The latter were shown to give prothrombin the  $Ca^{2+}$ -binding ability needed for its function. In contrast, abnormal prothrombin induced by VKA lacked Gla residues, explaining why prothrombin after VKA treatment failed to bind  $Ca^{2+}$  and lost its activity [77]. A year after the discovery of Gla residues in prothrombin, Esmon et al. [78], reported an enzymatic activity (vitamin K-dependent  $\gamma$ -glutamyl carboxylase) that catalysed the incorporation of  $CO_2$  into glutamic acid. These landmarks were followed by the identification of vitamin K epoxide reductase as the target enzyme of warfarin inhibition [79], the identification of a propeptide on the vitamin K-dependent protein for binding to  $\gamma$ -glutamyl carboxylase [80], and the discovery of a recognition site within the propeptide that is required for  $\gamma$ -carboxylation [81]. Recently, the gene encoding vitamin K epoxide reductase has been identified and cloned [82,83].

The components of vitamin K cycle include the following reactions (Figure 6) [70]: Vitamin K quinone is received from dietary sources and is reduced to vitamin K quinol ( $KH_2$ ) by vitamin K reductase (VKR; also called DT-diaphorase). Formed  $KH_2$  is a cofactor for  $\gamma$ -



## ***Mechanism of $\gamma$ -Carboxylation and epoxidation***

Both epoxidation of vitamin  $\text{KH}_2$  and  $\gamma$ -carboxylation of Glu represent unique biochemical reactions [85]. Whereas other carboxylation reactions are driven by ATP hydrolyses, the energy required for the carboxylation of Glu seems to be provided by a different approach [80,86]. The vitamin K-dependent  $\gamma$ -glutamyl carboxylase (GGCX) is an integral membrane protein, with a molecular weight of 94 kDa, and contains 758 amino acids with five transmembrane helix domains [70]. The human carboxylase gene is located in chromosome 2 at 2p12, consists of 15 exons with bordering introns, and is 13 kb in length [80]. A high degree of sequence homology has been found between human and rat (88%) and between human and bovine (94%) GGCX [80]. Recent discoveries of GGCX and Gla in several invertebrates, where blood coagulation is normally absent, suggest that the protein is evolutionarily older than the vertebrates [72]. Although it has been purified [87] and cloned [88], the structure of GGCX has yet not been determined by X-ray crystallography or NMR.

Vitamin K-dependent proteins contain a propeptide that plays a key role in directing these proteins to their carboxylation site on GGCX [80]. The propeptide contains a recognition site which binds GGCX [81]. The recognition site contains a Z-F-Z-X-X-X-A motif, where Z is an aliphatic amino acid, F is phenylalanine, A is alanine, and X is any amino acid [80]. Alteration of this motif, for instance by mutation, affects the carboxylation [70,89]. On the GGCX enzyme, 9-12 Glu residues at the amino terminus of each vitamin K-dependent protein are  $\gamma$ -carboxylated [90]. The site of carboxylation, called "Gla-domain", contains varying numbers of Glu residues, with as many as 12 Glu converted to Gla in human factor IX [91]. In prothrombin, all 10 Glu residues between 7 and 40 amino acids are carboxylated. Factors VII and X, as well as proteins C, S and Z, share homology with prothrombin Gla-domain, and undergo similar carboxylations [90]. The Gla domain and the  $\gamma$ -carboxylation recognition site are encoded by a single exon in the genes of vitamin K-dependent proteins [89].

Much of the current knowledge on the mechanism of  $\gamma$ -carboxylation is based on chemical reaction models. An attractive model, called "base-strength amplification", was postulated by Dowd and is widely accepted [90]. According to this model, the free energy of oxygenation of vitamin  $\text{KH}_2$  is used to transform a weak base to a strong base of vitamin K, capable of removing a proton from Glu. Briefly, the base-strength amplification model proposes the following: A weak base on the active site of GGCX removes a proton from vitamin  $\text{KH}_2$ . Then, vitamin  $\text{KH}_2$  becomes oxygenated, and an intermediary strong base of vitamin K is formed. The latter removes a proton from the  $\gamma$ -carbon of a Glu substrate. A reactive carbanion intermediate of Glu is then formed, which subsequently reacts with  $\text{CO}_2$  to form the Gla product [90]. Carboxylation and epoxidation reactions are tightly coupled, having a stoichiometry of 1:1 for both substrates and products [92]. Dowd and others suggested cysteine as the catalytic base that deprotonates  $\text{KH}_2$  [90]. Later studies, however, proposed that histidine is more likely to be the active site residue that initiates the carboxylation [85]. According to the base-strength amplification model, the oxygenation of vitamin K drives the carboxylation of Glu, and the access to vitamin K determines the rate of carboxylation [70,90]. Recent reports show also that the chaperone protein calumenin regulates the activity of  $\gamma$ -carboxylation system [73].

Once Gla-modified, vitamin K-dependent proteins leave the ER membrane and are further processed in the Golgi apparatus before they are secreted into the extracellular space [80]. Gamma-carboxylation provides coagulation factors with the  $\text{Ca}^{2+}$ -binding ability that they require for their binding to the negatively charged phospholipid membranes. It has been proposed that  $\text{Ca}^{2+}$ -binding causes a conformational change in these proteins, which facilitates their binding to phospholipid surfaces [72,93,94]. In the presence of calcium, the

conformational change results in an orientation of the hydrophobic side chains of residues Phe 4, Leu 5 and Val 8 in the Gla domain of prothrombin in such a way that an interaction with target phospholipid membranes is made possible. In the absence of calcium, these hydrophobic side chains are buried in the interior of the Gla domain [72]. Furthermore,  $\text{Ca}^{2+}$  ion is believed to bridge carboxylate groups on Gla residues and phosphate groups on the membrane [94].

### ***Vitamin K epoxide reductase***

In animals and humans, VKO formed in the  $\gamma$ -carboxylation reaction must be reduced back to vitamin K in order to enable a new carboxylation to take place. This vitamin K recycle is particularly important because the amount of vitamin K in the diet and its levels in the body are limited [70]. Historically, the enzyme that reduces VKO to vitamin K has been called vitamin K epoxide reductase complex (*VKOR*) or phylloquinone epoxide reductase. The Biochemical Nomenclature Committee of the International Union of Pure and Applied Chemistry (IUPAC) has assigned *VKOR* the Enzyme Commission number 1.1.4.1 (<http://www.chem.qmul.ac.uk/iupac/jcbtn/>) according to the following sub-branches:

#### *1. Oxidoreductase*

##### *1. Acting on the CH-OH group of donors*

##### *4. With a disulfide as acceptor*

##### *1. Vitamin-K-epoxide reductase (warfarin-sensitive)*

*VKOR* activity was first observed in 1970 [76], but all attempts to purify the enzyme from its ER membrane have failed and proved to be a difficult task. One of the reasons for this failure is believed to be that the enzyme complex is labile and sensitive to detergents and other chemicals used for the reconstitution trials [95]. Much about the nature of this enzyme has thus remained a matter of controversy. Some evidence suggested that *VKOR* is a multi-component enzyme with microsomal epoxide hydrolase and glutathione S-transferase as likely constituents of the complex [96]. Furthermore, the chaperone protein calumenin, mentioned above as a regulator for  $\gamma$ -carboxylase activity, has also been shown to inhibit the *VKOR* enzyme [84]. Calumenin has a  $\text{Ca}^{2+}$ -binding capacity and is embedded in the ER membrane. Its role in both  $\gamma$ -carboxylase and *VKOR* would thus add a further dimension to the regulation of blood coagulation [84].

A major breakthrough was recently achieved when two groups independently identified the gene encoding *VKOR* [82,83]. This discovery was preceded by the work of Kohn et al. [97], who had investigated warfarin resistance in rodents and had mapped the *VKOR* gene to rat chromosome 1. By using comparative ortholog mapping, they could then place the corresponding *VKOR* gene in mouse chromosome 7, and in human chromosome 10, 12 or 16. These findings were followed by the work of Fregin et al. [98], who mapped the defective gene (*VKOR*) causing combined deficiency of vitamin K-dependent clotting factors type 2 (*VKCFD2*) to human chromosome 16. These discoveries inspired Rost et al. [83] and Li et al. [82] to search the candidate *VKOR* gene in human chromosome 16. Rost et al. investigated a 4.0 Mb segment containing 129 genes on the short arm of chromosome 16. By positional cloning and linkage information from three species (human, rat, and mouse), they finally isolated a gene that extended over 5.1 kb and comprised three exons encoding a protein of

163 amino acids with a putative molecular mass of 18 kDa [83]. They associated this gene with two previously known human genetic disorders in the Online Mendelian Inheritance in the Man (OMIM) database: VKCFD2 (OMIM 607473) and warfarin resistance (WR; OMIM 122700). Cloning and overexpression of this gene showed a marked increase in *VKOR* activity, which was sensitive to warfarin inhibition [83]. The authors assumed that there might be other components of the enzyme, yet to be discovered [83], and they named their protein vitamin K epoxide reductase complex subunit 1 (*VKORC1*).

Li et al. [82] chose a different approach to identifying the *VKOR* gene. They started with the same region of chromosome 16, and selected 13 candidate genes of unidentified function that encode potential transmembrane proteins (understanding that *VKOR* is a transmembrane protein). Then, they used siRNA (short interfering RNA) technique against all of the 13 candidate genes to test their ability to inhibit *VKOR* activity in human cells. By this posttranscriptional gene-silencing technique, double-stranded short interfering RNAs are transfected into cells where they seek and target matching mRNAs for degradation [99]. From their knockdown trials, Li et al. found only one gene that was silenced by siRNA, and this resulted in a significant reduction of *VKOR* activity [82]. The silenced gene was identical to the *VKORC1* gene independently discovered by Rost et al. [83].

The gene identified by Rost et al. and Li et al., and its corresponding protein, has been given the official name *VKORC1* by the HUGO Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>), whereas the term *VKOR* refers to the historical protein that *in vivo* reduces VKO to vitamin K. This name separation is used to underline the possibility that other constituents in the putative *VKOR* complex might exist. There is, however, strong evidence indicating that *VKOR* is not a multiprotein complex and is hence identical to *VKORC1*. When Li et al., for instance, expressed *VKORC1* in insect cells that lacked vitamin K recycling ability, a full *VKOR* function was obtained [82]. It is, nevertheless, not inconceivable that *VKORC1* still might require some endogenous cofactors or coenzymes for its activity [95].

*VKORC1* shares a considerable sequence homology with a number of proteins found in animals, plants and bacteria [100]. The *VKORC1* gene and cDNA sequences are found at the NCBI web site (<http://www.ncbi.nlm.nih.gov/>) under the accession numbers AY587020 (for the gene) and AY521634 (for cDNA), respectively. The membrane topology of *VKORC1* has been investigated by Tie et al. [101] using different topology prediction programs. Most of these prediction algorithms have proposed three transmembrane helix domains flanked by four loops (Figure 7). N-terminus of the protein seems to locate in the ER lumen, whereas the C-terminus is probably in the cytoplasmic side. Four cysteines are absolutely conserved. Two of them (Cys43 and Cys51) are located on a cytoplasmic loop, whereas the two others (Cys132 and Cys135) are predicted to be buried in the third transmembrane helix nearest to the C-terminus [100] (Figure 7). A serine or threonine at position 57 on the cytoplasmic side is also conserved. These five residues are proposed to constitute the active site of *VKORC1* [100]. Residues Cys132 and Cys135 form a CXXC motif that is common for thioredoxin-like oxidoreductases. When these two cysteines were mutated *in vitro*, the activity of *VKOR* was completely eliminated [70,95,100]. These findings, and the fact that no natural mutation of these cysteines has been found in humans or rodents, strongly support the importance of the CXXC motif for *VKOR* function [95]. Furthermore, The CXXC motif has been proposed as a candidate for the thiol centre of the enzyme [84]. Its strictly conserved nature and its transmembrane location are also typical of thiol redox centres in other proteins. Buried in the third transmembrane helix is also a TYA motif where warfarin is predicted to bind. Both warfarin and vitamin K are hydrophobic molecules and partition into the phospholipid bilayer [95].

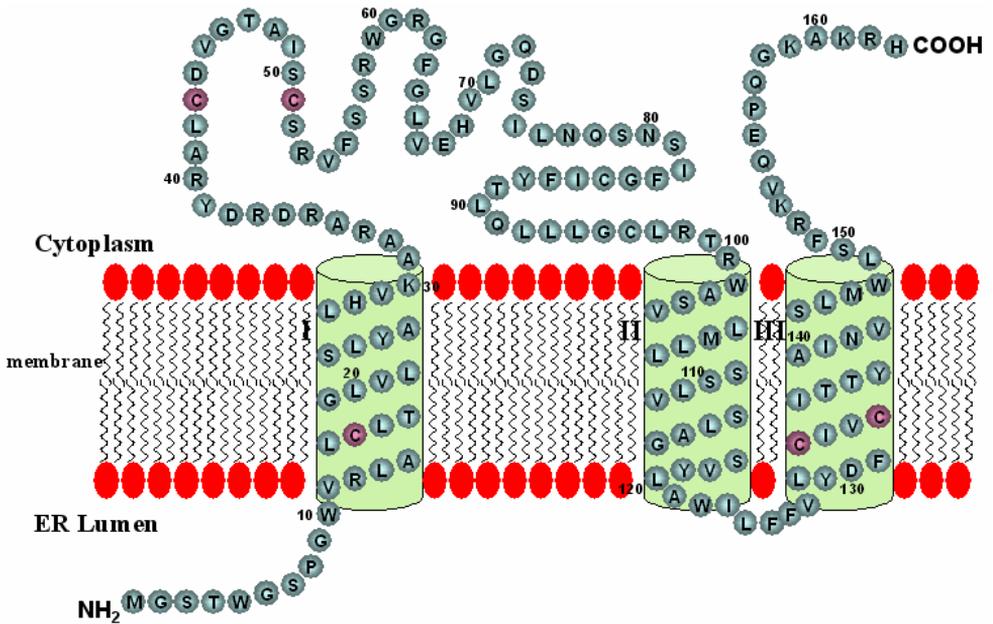


Fig. 7. Proposed membrane topology of *VKORC1*. Kindly provided by professor Darrel W. Stafford (University of North Carolina, USA).

### **Mechanism of warfarin action**

Whitton et al. emphasised in 1978 that warfarin and other VKA drugs act by blocking the function of *VKOR*. They also proposed that an unknown reducing agent was needed *in vivo* for the reduction of VKO to vitamin K. Since then, no sufficient data has been presented describing the mechanism of VKO reduction and warfarin inhibition. The best evidence, which also supports the existence of *in vivo* reducing agents, came in 1983 from Fasco et al. [102], who used rat liver microsomes and proposed a non-competitive inhibitory mechanism of warfarin. Their *in vitro* experiments revealed that a critical disulfide within the *VKOR* enzyme is oxidised during the reduction of VKO substrate. The disulfide group must then be regenerated to its reduced state in order to maintain an enzymatic activity. The authors suggested that warfarin binds covalently to the active site of *VKOR* and inhibits the enzyme by blocking further reduction of the critical disulfide group. The reducing agent needed for the regeneration of *VKOR* was dithiothreitol in the *in vitro* experiments of Fasco et al. [102]. The physiological electron donor needed for the reduction of *VKOR* thiol redox centre has not yet been identified. Some evidence points to NADH as the source of reducing equivalents via microsomal lipamide reductase [103]. Very recently, Wajih et al. [104] proposed a mechanism in which disulfide-dependent folding of reduced RNAase by protein disulfide isomerase (PDI) provides electrons for the redox centre in *VKOR* for reduction of VKO to vitamin K. The authors suggested also that PDI and *VKOR* form a tightly associated integral complex in the ER membrane, and that this association is necessary for the reduction of VKO. It remains, however, to be verified when *VKOR* is purified and reconstituted in phospholipid vesicles, a task which has proven very difficult so far.

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## Pharmacogenetics of warfarin

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Warfarin is a drug with narrow therapeutic range. The required dosing is highly variable and the treatment is regularly controlled with repeated analysis of PT-INR to ensure stable coagulation. A number of variables are believed to contribute to the observed inter-individual differences in dosage. Variations in dietary intake of vitamin K, interactions with other drugs involving pharmacodynamic or pharmacokinetic mechanisms, interactions with natural substances and herbal medicines, and age are all well-known factors that affect warfarin treatment [52,105,106]. However, over 40% of all inter-individual dose variations of warfarin are explained by genetic polymorphisms of two genes [107], as discussed below.

### ***CYP2C9* polymorphism**

Human *CYP2C9* enzyme is a heme-containing protein and a member of the Cytochrome P450 2C subfamily, including *CYP2C8*, *CYP2C18* and *CYP2C19*. It hydroxylates preferably substrates which are lipophilic and weakly acidic, including warfarin and many other drugs with narrow therapeutic index [108].

The gene encoding *CYP2C9* is located on the long arm of chromosome 10 (10q24.2) and is over 55 kb in length [109]. In humans, three allelic variants are found at significant frequencies: *CYP2C9*\*1 (Arg144/Ile359 - the wild type-allele), *CYP2C9*\*2 (Cys144/Ile359) and *CYP2C9*\*3 (Arg144/Leu359) [110]. Arg144 is encoded by exon 3 and is located in helix C, which is supposed to be part of the putative P450 reductase binding site of the protein [108]. Changed enzymatic function is thus expected when this residue is altered. Ile359 is located in exon 7 and maps on the proximity of the active site. Replacement of this amino acid leads to a change of  $V_{max}$  and  $K_m$  for *CYP2C9* substrates [108].

*CYP2C9* polymorphisms frequencies vary dramatically between different ethnic populations. Current data suggests that about two-thirds of the Caucasian and Turkish populations express the wild-type allele, whereas approximately one-third express either *CYP2C9*\*2 or *CYP2C9*\*3 alleles [109]. *CYP2C9*\*3 is found in all ethnic groups, with allelic frequencies of up to 10% in Caucasians and Canadian native Indians, up to 5% in Asians and 0.5 – 1.5% in African-Americans [111]. The *CYP2C9*\*2 allele is not detected in Asians (Chinese and Japanese), but is found in about 20% of Caucasians and 1 to 3.6% in African-Americans and Canadian native Indians, respectively [111]. However, the distribution of *CYP2C9* variants in populations of similar ethnicity is variable, and different frequencies have been reported for Caucasians. In Sweden, Yasar et al. [112] found that the frequencies of *CYP2C9*\*1, \*2 and \*3 for Swedish subjects were 82%, 11% and 7%, respectively. Furthermore, when populations of sub-Saharan Africa were compared, Scordo et al. found that Ethiopians were different from other black Africans with respect to *CYP2C9* polymorphisms and displayed higher allele frequencies for *CYP2C9*\*2 and *CYP2C9*\*3 (4.3% and 2.1%, respectively) than African-Americans or other black Africans [113].

The presence of *CYP2C9*\*2 and \*3 variants are associated with lower warfarin dose requirement and slower clearance of (S)-warfarin [110,114-117]. It has been reported that patients with these variants have approximately 40% reduced dose requirement compared with individuals expressing the wild-type allele (*CYP2C9*\*1/\*1) [111]. It is also assumed that a gene-dose effect exists for these variants, with homozygous \*2 and \*3 requiring the lowest dose, heterozygotes as intermediates and \*1 as the reference allele [51]. In the initiation phase

of warfarin treatment, patients with homozygous \*2 and \*3 variants are known to be associated with excessive anticoagulations, particularly among elderly individuals [34].

### ***VKORC1* polymorphism**

Polymorphisms affecting the sensitivity of *VKORC1* for warfarin is a relatively new discovery and has been observed following the identification of the *VKORC1* gene by two research groups [82,83]. Rost et al. reported warfarin resistant patients with single-nucleotide exchanges in *VKORC1* [83]. Three of these patients had a dose requirement of 220 – 250 mg warfarin/ week, which is considered unusually high. Two other patients did not respond to warfarin for all doses tested [83]. This observation was followed by the report by Harrington et al., who found a Val66Met transition in the *VKORC1* protein causing warfarin resistance [118]. These point mutations were found in the coding regions (exons) of the *VKORC1* gene. They are, however, rare and not found in the general population, and can therefore not explain the high degree of inter-individual variation in warfarin dose requirement.

The mutations in *VKORC1* that mostly influence warfarin dosage are found in the noncoding regions of the gene [107,119-121]. These common single-nucleotide polymorphisms (SNPs) show inter-ethnically different distributions and have a greater impact on dose requirement than do *CYP2C9* variants [107,119]. The first identified SNPs in the *VKORC1* gene were reported by D'Andrea et al., who studied two SNPs at nucleotide positions 6484 and 9041 [121]. The 6484 SNP is located in intron 1 (Figure 8) and has a CC genotype for the wild-type allele, but can be found as CT or TT allelic variants. Patients with the CC allele were generally found to require a higher (6.2 mg/day) warfarin dose requirement than patients carrying the CT (4.8 mg/day) or TT (3.5 mg/day) alleles. The 9041 polymorphism is found in the 3'untranslated region (UTR) of the gene (Figure 8) and is involved in a G>A transition, where the AA genotype is associated with increased warfarin dosage [121]. Rieder et al. identified 10 common noncoding SNPs forming two main haplotype groups: A (low dose) and B (high dose) [119]. They found that the A haplotype has a frequency of nearly 89% in Asians, 37% in Caucasians, and only 14% in Africans. This difference in haplotype distributions matched the differences in warfarin dosage between the different ethnic populations.

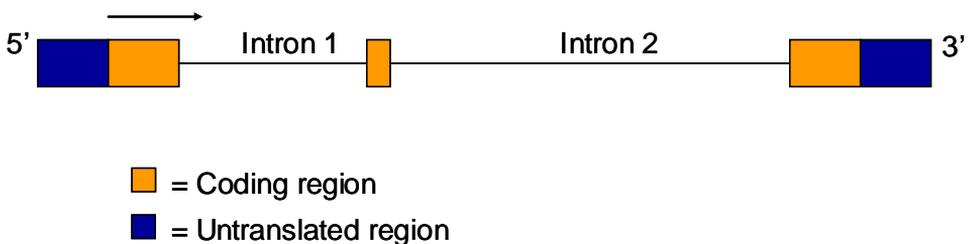


Fig. 8. Graphic illustration showing the organisation of *VKORC1* gene. Three exons code for the primary structure of the protein. Untranslated regions (5' and 3') containing putative regulatory sequences are also shown.

A more comprehensive haplotype map involving 28 *VKORC1* polymorphisms was reported by Geisen et al. [120]. Their study confirmed the previous observation of Rieder et al. [119] and further extended the number of main haplotypes to four. They called their haplotypes

*VKORC1*\*2 (corresponding to the low dose A haplotype of Rieder), *VKORC1*\*3 and *VKORC1*\*4 (Both correspond to the high dose B haplotype of Rieder) (Table 2). A fourth variant, *VKORC1*\*1, was also identified as the ancestral haplotype and was found only in Africans [120]. These main haplotypes were suggested to cover almost all of the *VKORC1* genetic variability. In populations of Asian ethnicity, *VKORC1*\*2 has been found to be the dominating haplotype, corresponding to 90% in Chinese Americans [119], 86% in Hong Kong Chinese [122], and 89% in Japanese [123]. In Europeans, *VKORC1*\*2 and *VKORC1*\*3 are the principal variants (about 40% each) [120], whereas Africans have a frequency of 14% for *VKORC1*\*2, 31% for *VKORC1*\*1, and 43% for *VKORC1*\*4. Haplotype *VKORC1*\*4 is rare in Asians but is more common in African and European populations [120]. These findings suggest that the populations in Asia (excluding subcontinental India and the Middle East) generally require lower doses of warfarin than African and European populations. The present data confirms that this is the case [119,122,123].

Wadelius et al. [107] have estimated that the noncoding polymorphisms in *VKORC1* explain approximately 30% of the variations in warfarin dose requirement when factors such as *CYP2C9* polymorphisms, age, bodyweight and drug interactions were excluded. Sconce et al. [124] proposed a dosing regimen for warfarin where the contribution of age, body size, *CYP2C9* and *VKORC1* polymorphisms were investigated. They found that the total impact of these factors on warfarin dose variability was nearly 55%. Altogether, it is becoming clear that *VKORC1* is an important genetic determinant for warfarin dose requirement.

Table 2. The most common SNPs with minor allele frequency of >5% identified in the human *VKORC1* gene and the haplotypes they segregate.

Gene Region	Nucleotide position	Nucleotide exchange	Haplotype name (Geisen et al.)	Haplotype name (Rieder et al.)	Effect on warfarin treatment	Reference
Promoter	3673	G>A	<i>VKORC1</i> *2	Group A	Low dose	[119]
Intron 1	5808	T>G	<i>VKORC1</i> *2	Group A	Low dose	[119]
Intron 1	6484	C>T	<i>VKORC1</i> *2	Group A	Low dose	[121]
Intron 2	6853	G>C	<i>VKORC1</i> *2	Group A	Low dose	[119]
Intron 2	7566	C>T	<i>VKORC1</i> *2	Group A	Low dose	[119]
Intron 2	8026	A>G	<i>VKORC1</i> *3	-	High dose	[120]
3'UTR	9041	G>A	<i>VKORC1</i> *3	Group B	High dose	[121]
Intron 1	6009	C>T	<i>VKORC1</i> *4	Group B	High dose	[119]

The molecular mechanisms that elucidate how *VKORC1* polymorphisms interact with warfarin have yet not been described. Both Wadelius [107] and Rieder [119] speculated that the observed association between polymorphism and dose variation could be due to possible effects of these SNPs on mRNA transcription, splicing or stability. Rieder et al. observed that the expression of *VKORC1* mRNA varied depending on haplotype form. They found that the A low dose haplotype (*VKORC1*\*2) had almost 3 times lower mRNA expression levels than

the B variant (*VKORC1*\*3 and *VKORC1*\*4) and that this might explain the lower dose requirement. Bodin et al. [125] identified a potential nuclear factor 1 (NF1) binding site motif in the *VKORC1* promotor at position -1639 upstream of the ATG initiation site (nucleotide position 3673). A possible explanation would thus be that the -1639G>A substitution alters the binding site for NF1 and consequently affects *VKORC1* transcription efficiency. Furthermore, Takahashi et al. recently reported that a haplotype combination including *VKORC1* 1173C>T (*VKORC1*\*2) was associated with an altered correlation between the concentration of unbound (S)-warfarin and INR in Japanese and Caucasian patients [123]. Finally, our retrospective analysis of case records has revealed that *VKORC1*\*2 is associated with difficulties to attain stable therapeutic PT-INR in patients undergoing anticoagulation therapy [126]. In contrast, *VKORC1*\*3 and *VKORC1*\*4 haplotypes were less associated with these complications. Current reports therefore suggest that the low dose *VKORC1*\*2 variant is more important than its high dose analogues for correct warfarin dosage.

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## Chromatography of warfarin

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Determination of plasma warfarin has often been performed by using high-performance liquid chromatography (HPLC). One of the earliest HPLC methods for detection of warfarin was reported in 1974 by Vesell et al. [127], who used an achiral, reversed phase column packed with octadecylsilane sorbent, and a mobile phase consisting of 10% dioxane in 90% water. Under those conditions, a single peak of warfarin was separated from its 7-hydroxywarfarin metabolite. A more sensitive, achiral method, with a limit of detection (LOD) of 60 ng/mL, and involving an extraction from acidified plasma was later reported by Robinson et al. [128]. However, these achiral methods were insufficient and could not constitute a tool for pharmacokinetic studies. To understand the metabolism and elimination of warfarin in living systems, stereo-specific detection of (R)- and (S)-isomers is needed.

A method using a bovine serum albumin chiral stationary phase (BSA-CSP), coupled with a Pinkerton internal-surface reversed phase (ISRP) achiral column, was published in 1988 by Chu and Wainer [129]. Although a LOD of 25 ng/mL was obtained for both (R)- and (S)-warfarin, this method suffered a poor chromatographic resolution and relatively high capacity factors (for HPLC theory and terminology, see Appendix). A new generation of stereo-specific warfarin detection was presented by Naidong and Lee in 1993 [130], who used a silica-bonded  $\beta$ -cyclodextrin column with a polar solvent mobile phase and naproxen as internal standard. With UV detection at 320 nm, the separation was good, with baseline resolution for both (R)- and (S)-warfarin. However, naproxen is a common analgesic drug and can be detected in human plasma from individuals who have taken the drug, making it unsuitable as internal standard [131]. More recently, other methods using Pirkle stationary phases and other internal standard calibrations have been reported [43,131-133]. Table 3 summarises the different HPLC methods found in the literature for the quantification of warfarin. Most of these methods used UV absorbance or fluorescence detectors. Two methods [134,135] utilised mass spectrometry as a detection technique.

Table 3. The different HPLC methods found in the literature for the determination of plasma warfarin.

Author	Reference	Column/ stationary phase	Type	Internal standard	Detection
Banfied and Rowland	[136]	Pre-column diastereoisomeric formation	Semi-chiral	None	Fluorescence
Robinson et al.	[128]	Bondapak C-18	Achiral RP*	None	UV absorbance
Chu and Wainer	[129]	BSA – ISRP	Chiral RP	Benoxaprofen	Fluorescence
McAleer et al.	[137]	AGP	Chiral RP	Naproxen	Fluorescence
Naidong and Lee	[130]	$\beta$ -Cyclodextrin	Chiral RP	Naproxen	UV absorbance
Takahashi et al.	[138]	Chiralcel OD cellulose-derived column	Chiral NP**	Naproxen or diclofenac	UV absorbance or Fluorescence
Henne et al.	[133]	Pirkle (R,R) Whelk-O 1	Chiral RP	Racemic ethylwarfarin	UV absorbance
Ring and Bostick	[131]	$\beta$ -Cyclodextrin	Chiral RP	Oxybenzone	UV absorbance
Rentsch et al.	[132]	Pirkle (R,R) Whelk-O 1	Chiral NP	n.a. ¶	UV absorbance
Boppana et al.	[139]	Ovomucoid silica column	Chiral RP	p-chloro warfarin	Fluorescence
Lombardi et al.	[57]	Symmetry shield column	Achiral RP	None	UV absorbance
Locatelli et al.	[140]	AGP	Chiral RP	Naproxen	UV absorbance
Osman et al.	[43]	Pirkle (R,R) Whelk-O 1	Chiral-RP	Oxybenzone	UV absorbance
Sun et al.	[141]	C18	Achiral RP	Naproxen	UV absorbance
Ufer et al.	[134]	Luna C18	Achiral RP	deuterium-labeled 7-hydroxywarfarin	Mass spectrometry (LC/MS)
Naidong et al.	[135]	$\beta$ -Cyclodextrin	Chiral-RP	p-chloro warfarin	Mass spectrometry (LC/MS)

\* RP = Reversed phase

\*\* NP = Normal phase

¶ (R) and (S) warfarin were used as internal standard for the determination of phenprocoumon and acenocoumarol.

# AIMS

The aims of this thesis were:

- To develop a cost-effective, sensitive and stereospecific HPLC method for the determination of warfarin enantiomers in human plasma.
- To investigate the relationship between *VKORC1* variants and warfarin treatment, particularly the effect of the common *VKORC1* polymorphisms on required dose, plasma concentrations of (S)- and (R)-warfarin, and the stability of PT-INR for treated patients.
- To study whether the S/R-ratio of total plasma warfarin varies with *VKORC1* common polymorphisms, and whether this is correlated with *CYP2C9* variants or other factors.
- To find possible causes behind the differences which are observed between the different methods used for the measurement of PT-INR.

# MATERIALS AND METHODS

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## Introduction to methods and techniques

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### ***Chiral stationary phases***

#### **CYCLODEXTRINS**

Cyclodextrins (CD) are oligosaccharides forming cyclic structures containing 6 – 12 D(+) glucopyranose units bonded together by alpha-(1,4) linkages. Due to steric hindrance, fewer than 6 units cannot be formed.  $\beta$ -CD contains 7 glucose units with a cavity diameter of 6 – 8 Å. The CDs are linked to a silica carrier gel through a spacer arm. They contain hydrophilic outer surfaces and a hydrophobic cavity where hydrophobic molecules are entrapped. This entrapment/inclusion takes place without the formation of formal chemical bond [142].

#### **ALPHA- ACID GLYCOPROTEIN**

Another chiral phase that has been utilised for warfarin enantiomeric separation is  $\alpha$ -acid glycoprotein (AGP) [137,140]. AGP is a chiral selector which is immobilised on spherical 5  $\mu$ m silica particles. AGP columns are usually run in reversed phase mode and can be used for direct resolution of enantiomers without derivatisation. The enantiomers bind to the binding domain of the protein by either electrostatic or non-electrostatic interactions [143]. Selectivity and capacity factors can be adjusted by a change of the mobile phase composition or by changing the pH [143].

#### **PIRKLE-TYPE CHIRAL COLUMN**

These stationary phases bear the name of Bill Pirkle, who in the 1970s and 1980s developed a series of silica covalently bonded chiral phases with improved chromatographic resolution [144,145]. The Pirkle chiral selectors are relatively small molecular weight substances with few chiral centres. A large number of groups are, however, bonded to the silica, and the total number of chiral centres available in the column is therefore sufficient to resolve a wide range of different analytes. The advantage of Pirkle-type columns is that the overall interacting molecule is small and is covalently bonded to silica carrier gel, giving stability and longer column life.

The Pirkle stationary phases generally form three main classes:  $\pi$ -electron acceptors,  $\pi$ -electron donors, and the  $\pi$ -electron acceptor/ $\pi$ -electron donors. Major binding sites are

categorised as  $\pi$ -acidic or  $\pi$ -basic aromatic rings, acidic or basic sites, and steric interaction sites [144]. Aromatic rings are usually potential sites for  $\pi$ - $\pi$  interactions. Acidic sites provide hydrogens for intermolecular hydrogen bonds. Such hydrogen is often an amido proton (N-H).

A type of Pirkle column used for the determination of warfarin is the Whelk-O 1, which is a  $\pi$ -electron acceptor/ $\pi$ -electron donor phase containing 1-(3,5-Dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene covalently bonded to silica (Figure 9).

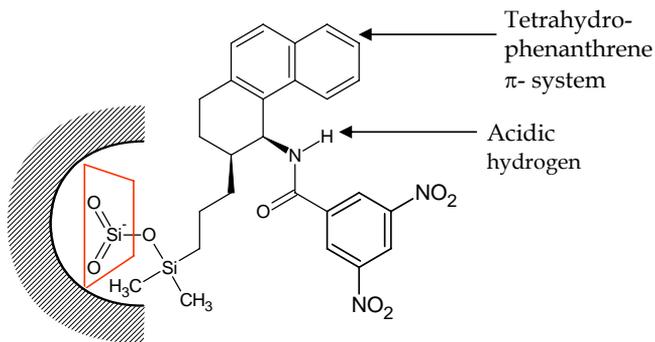


Fig. 9. A diagram showing the key functional groups involved in the chiral recognition of Pirkle Whelk-O 1 column.

## Denaturing HPLC

Denaturing HPLC (DHPLC) is a relatively new technology, developed in the last decade, and is used to screen DNA variations and in allele discrimination studies [146]. DHPLC takes advantage of the fact that double-stranded short DNA fragments containing a single-base mismatch have different retention profiles than correctly matched DNA when the nucleic acids are partially denatured. The DNA fragments are prepared by PCR (Polymerase Chain Reaction), and prior to chromatography they are denatured and reannealed. Individuals who are heterozygous for a SNP have a ratio of 1:1 between mutant and wild-type DNA. When the DNA fragments are heated to 95°C, and then slowly cooled down, they reanneal and form a mixture of hetero- and homoduplexes (Figure 10). The heteroduplex DNA fragments contain a higher proportion of single-stranded DNA and are consequently more loosely attached in the column than their homoduplex analogues.

DHPLC is run in a reversed phase mode and uses hydrophobic columns, such as Transgenomic's DNASep® cartridges. However, such columns have normally no affinity for the negatively charged DNA molecules and require ion pairs that form bridges for the adsorption of DNA fragments to the sorbent particles. Triethylammonium acetate (TEAA) is commonly added in the mobile phase as an ion-pairing molecule. Two different interactions take place in the column. One is an electrostatic interaction between the negatively charged phosphate groups of DNA and the positively charged ammonium ion of TEAA. The other is a hydrophobic interaction between the alkyl chains of the ion-pairing molecule and the stationary phase. Like in ion-exchange chromatography, elution of the sample is

accomplished by a gradient flow, for instance by increasing the concentration of acetonitrile ( $\text{H}_3\text{C}-\text{C}\equiv\text{N}$ ) in the elution fluid. The content of G-C base pairs, the temperature, and the presence of hetero- or homoduplexes are factors that determine the retention time of DHPLC .

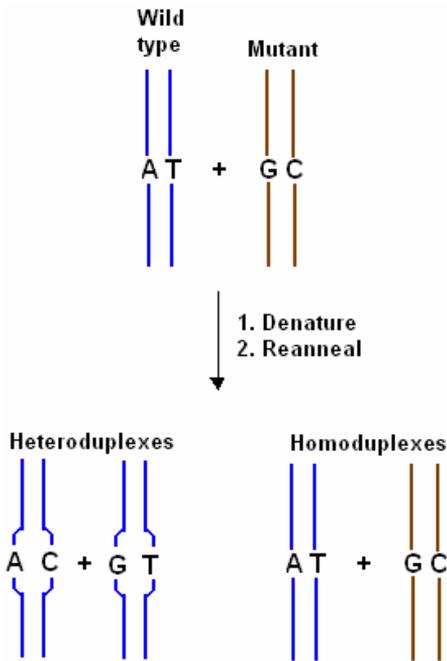


Fig. 10. A diagram illustrating hypothetical DNA fragments from a heterozygous individual analysed by DHPLC. One of the homologous chromosomes contains a single-nucleotide mutation, whereas the other has the wild allele. Denaturing followed by reannealing produces a mixture of hetero- and homoduplexes.

### DNA sequencing with MegaBace

DNA sequencing with MegaBace is based on the chain termination method using dideoxynucleotide triphosphate (ddNTP). DNA strands are normally elongated with dinucleotide triphosphate (dNTP) with a free OH-group on the 3' carbon of each nucleotide (A, T, G and C). On ddNTPs, this OH-group is replaced by a hydrogen atom and, as a result, the sequencing reaction is terminated when a ddNTP is incorporated. In MegaBace DNA sequencing, each ddNTP is labelled with two dyes, of which one is a fluorescein dye (donor), which absorbs light energy from a laser beam, and transfers the energy to the other dye (acceptor), which in turn emits light. The different ddNTPs (ddA, ddT, ddG and ddC) have specific and different acceptor dyes, and the emitted light sent by each ddNTP is therefore characteristic for that nucleotide.

The chain termination by ddNTPs occurs randomly, and fragments of different lengths are produced. These are separated on capillary array electrophoresis using narrow-bore capillaries filled with a separation matrix to separate DNA fragments. The separation depends on the size of the DNA molecules, with shorter fragments moving faster than longer molecules. Finally, the samples are scanned by the instrument, and the order of the sequence is read by identifying each terminated fragment.

## DNA sequencing with Pyrosequencing

In pyrosequencing, incorporated DNA bases are detected in real-time during the sequencing reaction [147,148]. The sample, which is normally a PCR product of a short DNA sequence, is first treated with NaOH to prepare an ssDNA, which is then hybridised with a sequencing primer. A reagent containing enzymes (polymerase, apyrase, sulfurylase and luciferase), the four nucleotides (dNPTS) and the substrates (adenosine 5' phosphosulfate (APS) and luciferin) is also prepared. The nucleotides are sequentially added to the reaction plate, and if a base is complementary to the DNA template, the polymerase enzyme catalyses the incorporation of that base, and the primer is elongated with one base. At the same time a pyrophosphate (PPi) is released (Figure 11). Released PPi, together with the substrate APS, is used by sulfurylase to generate ATP. Formed ATP, in turn, is utilised by luciferase, which converts the luciferin substrate to oxyluciferin. The latter generates light in amounts that are proportional to the amount of ATP, which in turn has an equimolar relationship with incorporated nucleotides. A CCD camera finally detects the light generated by oxyluciferin, and a pyrogram (peak) is presented by a computer. The magnitude of the light signal is proportional to the number of nucleotides incorporated in the reaction. Before another nucleotide is added, excessive dNTPs and ATP are degraded by apyrase (Figure 11).

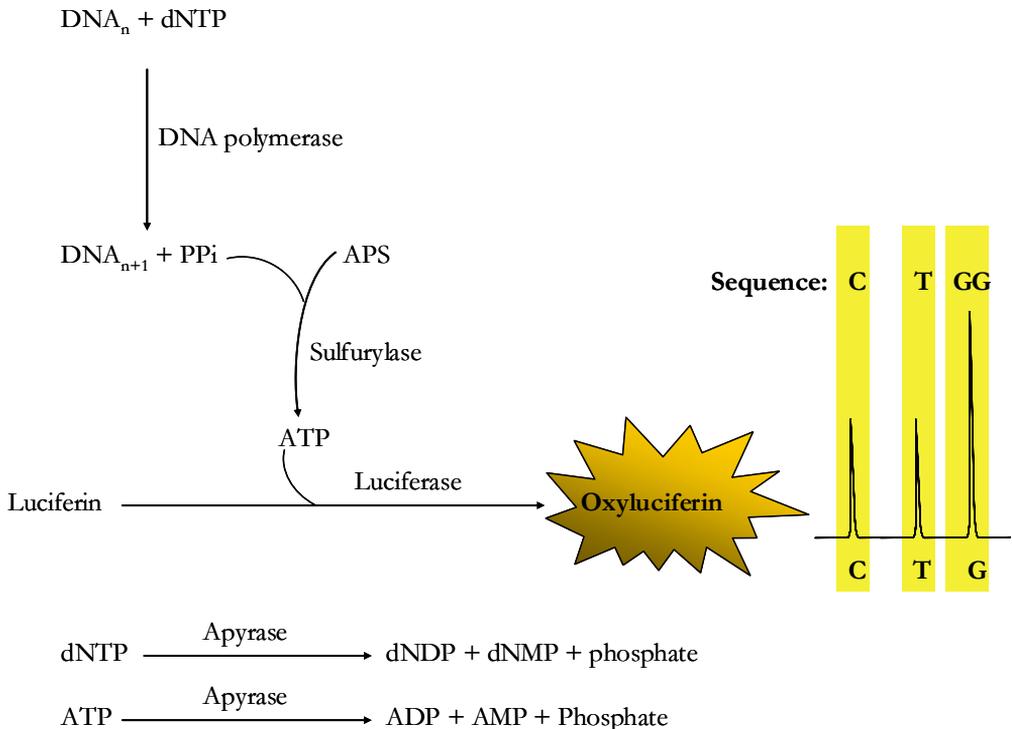


Fig. 11. Principle of the pyrosequencing reactions leading to the generation of light in the form of oxyluciferin, and the real-time detection of incorporated nucleotides presented as pyrograms.

## ***Polymerase Chain Reaction (PCR)***

PCR, a universal technique with many applications, is used to enzymatically duplicate DNA. It was introduced by Kary Mullis in the mid 1980s [149] and has since become an essential tool in every laboratory in which some form of genetic analysis is carried out. With PCR, any region of DNA can be copied by using a reagent containing primers (short single-stranded DNA), DNA polymerase, dNTPs, magnesium and optimised buffer. Heat recycling is also required for the process. PCR consists of cycles that are repeated for several times (e.g. 15 – 50x). Each cycle contains three different steps: denaturation, annealing and extension. In denaturation, DNA molecules are heated at 94–95 °C to break the chemical bonds between the two antiparallel strands of DNA. Two single-stranded DNA (ssDNA) are consequently formed. In annealing, the sample is cooled down to allow a pair of primers to bind, each being complementary to one ssDNA. The annealing temperature is variable and depends on the nature of the template DNA and the melting temperatures of primers. Temperatures of 50 – 60 °C are commonly used. In the extension step, the heat is increased to 72 °C to allow the amplification reaction to take place. The cycle is subsequently rounded off and a new cycle can start. The number of copies formed after  $n$  cycles of PCR becomes  $2^n$ . After 35 cycles, for instance,  $2^{35}$  or 34 billion copies of DNA are generated.

The Taq polymerase used in PCR is stable at high temperatures. The PCR process is thus automated and operated by a thermal cycler that heats and cools the reaction tubes (or plates) at the temperature required for each step. Once the reaction is finished, the copied DNA material containing a selected region of a gene is analysed on agarose gel electrophoresis to estimate the quality of the sample. PCR products can then be used to carry out sequencing or genotyping investigations.

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## Study design

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### **Patient samples**

Blood samples were collected from anticoagulated patients at anticoagulation clinics of the hospitals in Linköping, Eksjö, Motala, Värnamo and Västervik (Papers I–III) or were obtained from *Laboratorieservice* department, Laboratory Medicine, University Hospital in Linköping (Paper IV). For DNA and warfarin analysis, the samples were collected in 5-mL evacuated tubes containing EDTA. For coagulation analysis 2.7-mL tubes containing 0.5 mL of 0.13 mol/L buffered trisodiumcitrate were used. Ethical permission was obtained from the regional board of Ethical Review (Linköping, Sweden).

### **Calibrators and controls**

Plasma for calibrator and quality control (QC) samples used for warfarin analysis (Paper I) was collected from healthy blood donors and was obtained from *Blodcentralen*, Laboratory Medicine, University Hospital in Linköping. DNA samples ( $n = 180$ ), used as controls for population and allele frequency studies (Paper II), were collected from randomly selected individuals from the south-eastern region of Sweden. QC samples used for PT methods (Paper IV) were purchased from Medirox (Nyköping, Sweden) at two levels: NKP (normal control plasma) and OKP (abnormal control plasma).

### **Inclusion and exclusion criteria**

Inclusion criteria were:

- Informed consent.
- For patients, only those individuals who had stable anticoagulation over a period of time were included in the study (Papers I–III).
- For controls, only healthy individuals were included in the study.

Exclusion criteria were:

- Rejection of informed consent.
- Failure to analyse the sample or failure to pass the quality control in result assessment.
- For Papers I–III, failure to show warfarin enantiomers in plasma (patient samples), i.e. insufficient compliance.
- Drug interactions (provided they were known).

## **Statistical methods**

For warfarin chromatography (Paper I), regression analysis was used to validate the calibration graphs and the linearity of the method. Relative standard deviations (RSD) were calculated to evaluate the intra-day and inter-day precision of the method. The bias for calculated mean warfarin concentrations of controls was determined to find the deviation from the nominal plasma concentration. Absolute correlation distance method of Pearson was used to assess the relationship between warfarin and PT-INR and between (S)- and (R)-warfarin.

For *VKORC1* genotyping (Paper II), deviation of the studied SNPs from Hardy-Weinberg equilibrium was investigated with chi-squared test using a significance level of  $P < 0.05$ . Mann-Whitney *U* test was used for tests containing two groups. Kruskal-Wallis test was employed for groups of more than two. Inter-quartile illustration was performed with Box and Whisker plots.

For warfarin S/R-ratios and their relation to *VKORC1* polymorphism (Paper III), non-parametric analysis containing median test, Two-Sample Kolmogorov-Smirnov test or Mann-Whitney *U* was used at a 2-tailed significance level of  $P < 0.05$ .

For studies on PT methods (Paper IV), the absolute correlation distance method of Pearson was used to investigate the relationship between the different reagents.

## **Retrospective study**

A retrospective analysis was carried out on patient records to investigate the effect of different common *VKORC1* polymorphism on warfarin treatment (Paper II). Records of stable anticoagulated patients from Linköping and Motala, which were available via the data system of the anticoagulation clinic in Linköping, were studied. Two different periods were considered: The *initiation time*, defined as the first four weeks of treatment, and the *latest 12 months* during which each patient was regularly anticoagulated. The two periods did not overlap in any case. If confounding factors, such as drug interactions, were found, the exclusion principle was applied. The study focused on three *VKORC1* variants: *VKORC1*\*2, *VKORC1*\*3 and *VKORC1*\*4. Only those patients who were homozygous for these variants were considered. INR values for each patient, both in the initiation phase and in the latest 12 months of treatment, were collected to calculate the coefficient of variation (CV), number of visits and the number of INR values outside the therapeutic range. Age and gender were also recorded.

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## Experimental Procedures

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Analytical and technical details are available in Papers I–IV and are not further discussed in this section. Only general experimental procedures are considered below.

### ***Determination of warfarin by HPLC (Paper I)***

Stem and working solutions of racemic warfarin containing approximately equal amounts of (S)- and (R)-isomers were prepared. A working solution of oxybenzone used as internal standard (IS) was also prepared. Warfarin and IS solutions were dissolved in methanol and were kept at  $-20^{\circ}\text{C}$ . A chiral column of type Pirkle (R,R) Whelk–O1 formed the core of the chromatographic system, which furthermore contained a Hypurity C4 guard cartridge, Waters 616 gradient pump, a Gilson 230 XL autosampler and a Waters 996 photo diode array detector (PDA). Before chromatography, patient and control samples as well as calibrators were extracted by addition of sulphuric acid followed by liquid-liquid extraction with ethyl acetate and centrifugation. During the method development, ether and ethyl acetate were tested as organic solvents, but the latter was chosen because some interferences were detected in ether extracts. The mobile phase contained methanol/acetonitrile/water (50/10/40, v/v) with 0.1% glacial acetic acid. The proportion of acetonitrile was critical for the selectivity. The PDA detector scanned absorbance at wavelengths 200 – 400 nm, with 305 nm being the extracted wavelength. In the validation step, linearity, LOD, LOQ, recovery and precision of the method were determined from spiked plasma.

### ***Haplotype analysis of VKORC1 (Paper II)***

Genomic DNA of 98 patients was extracted from whole blood on GenoVision 48 BioRobot using Magnetic beads according to the instruction of the manufacturer (Qiagen, Hilden, Germany). The sequences to be studied were amplified by PCR. Mutation analysis was carried out with DHPLC on a Wave DNA fragment analysis system (Transgenomic Inc., Omaha, NB, USA). A DNA Sep HT column was utilised to separate PCR fragments, using a linear acetonitrile gradient (0.1 M TEAA / 0.1 M TEAA with 25% acetonitrile). A MegaBACE™500 instrument (Amersham Biosciences, Piscataway, NJ, USA) confirmed potential mutations and polymorphisms. Genomic sequence for *VKORC1* was downloaded from the National Center for Biotechnology Information (GenBank acc. No. AY587020 <http://www.ncbi.nlm.nih.gov/>). The previous work of Geisen et al. [120] was used to identify *VKORC1* haplotypes using 14 biallelic markers. Warfarin analysis was performed as above (Paper I). Finally, a retrospective analysis was carried out as described above.

### ***Warfarin S/R-ratios and their relation to VKORC1 common polymorphism (Paper III)***

This study was a continuation of Paper II. The question at issue was whether warfarin S/R-ratio varies with *VKORC1* haplotypes and whether this was correlated with *CYP2C9*\*2 and \*3 variants. To investigate this matter, all patients with homozygosity to *VKORC1*\*2, *VKORC1*\*3 and *VKORC1*\*4 and whose plasma warfarin were previously determined (Paper II) were genotyped for *CYP2C9*\*2 and *CYP2C9*\*3 variants. Genotyping was performed on a pyrosequencing instrument (Biotage AB, Uppsala, Sweden) according to the method

previously described by Eriksson et al. [150] with some modifications (see Paper III). Prior to pyrosequencing, genomic DNA was extracted as above (Paper II) and target DNA sequences (*CYP2C9*) were prepared with PCR. S/R-ratios of warfarin for the different *VKORC1* variants were then compared. The patients were categorised to low dose (*VKORC1*\*2) and high dose haplotype groups (*VKORC1*\*3 and *VKORC1*\*4). Statistical calculations were carried out to study the relationship between *VKORC1* haplotype and warfarin S/R-ratio, and between *CYP2C9* variant and warfarin S/R-ratio.

#### ***Studies on PT methods (Paper IV)***

This study examined the basic causes for the observed large variations between the different PT methods. Patient and quality control samples were analysed undiluted and 2×, 7×, 10×, and 12× prediluted in buffer. Bovine vs. human depleted plasmas and rabbit brain vs. human placenta thromboplastins were compared, and the correlations between the different reagents were investigated. PT analysis was carried out on an ACL Futura (Instrumentation Laboratories, Milan, Italy) according to the instructions of the manufacturer.

# RESULTS

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## Paper I

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The purpose of this work was to develop a rapid, reproducible and sufficiently sensitive method for the measurement of plasma warfarin. Two different chiral selectors were tested. A column containing cyclodextrin was used in the early stages of method development but was later replaced by a Pirkle Whelk-O1 column. The latter offered better stability and reproducibility. Method linearity was evaluated to determine the range in which the calibration curves were valid. Both (S)- and (R)-warfarin showed linearity up to 10  $\mu\text{g/mL}$ , which is the range in which most patient samples are expected to lie (Figure 12). The recovery of warfarin was estimated at not less than 80%, and Oxybenzone (IS) coextracted well with warfarin. The detection limit of the method was 16 ng/mL for (S)-warfarin and 18 ng/mL for (R)-warfarin. Separation and retention profiles of warfarin and IS are shown in a typical patient chromatogram (Figure 13). There was a low correlation between warfarin and PT-INR ( $r = 0.23$ ,  $y = 0.3044x + 0.9712$ ), which is expected because plasma warfarin levels or warfarin dose are known not to significantly correlate with the pharmacodynamic response of the drug.

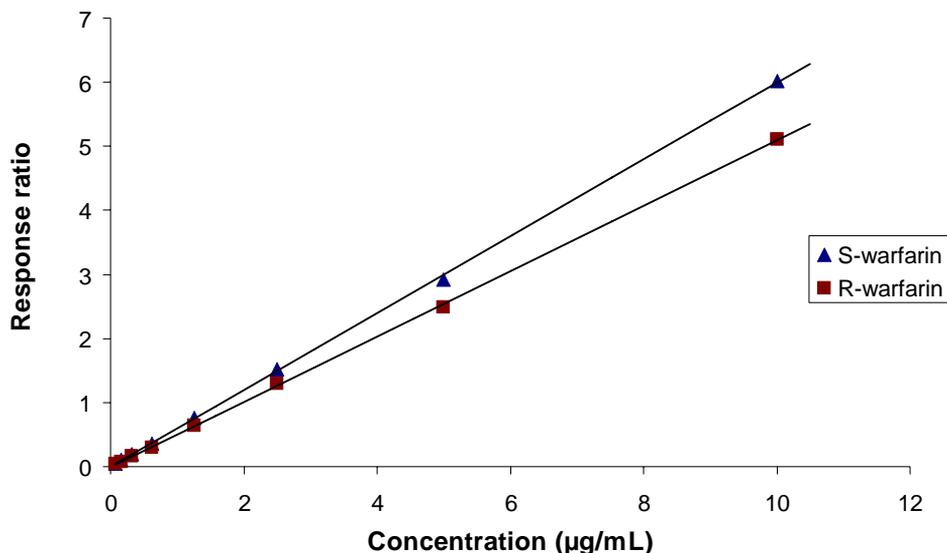


Fig. 12. Linearity of warfarin calculated from spiked plasma ( $R^2 = 0.9997$ ,  $y = 0.5995x - 0.003$  for (S)-warfarin, and  $R^2 = 0.9998$ ,  $y = 0.5098x - 0.0054$  for (R)-warfarin). Each graph contains values of eight points between 0.078 and 10  $\mu\text{g/mL}$ .

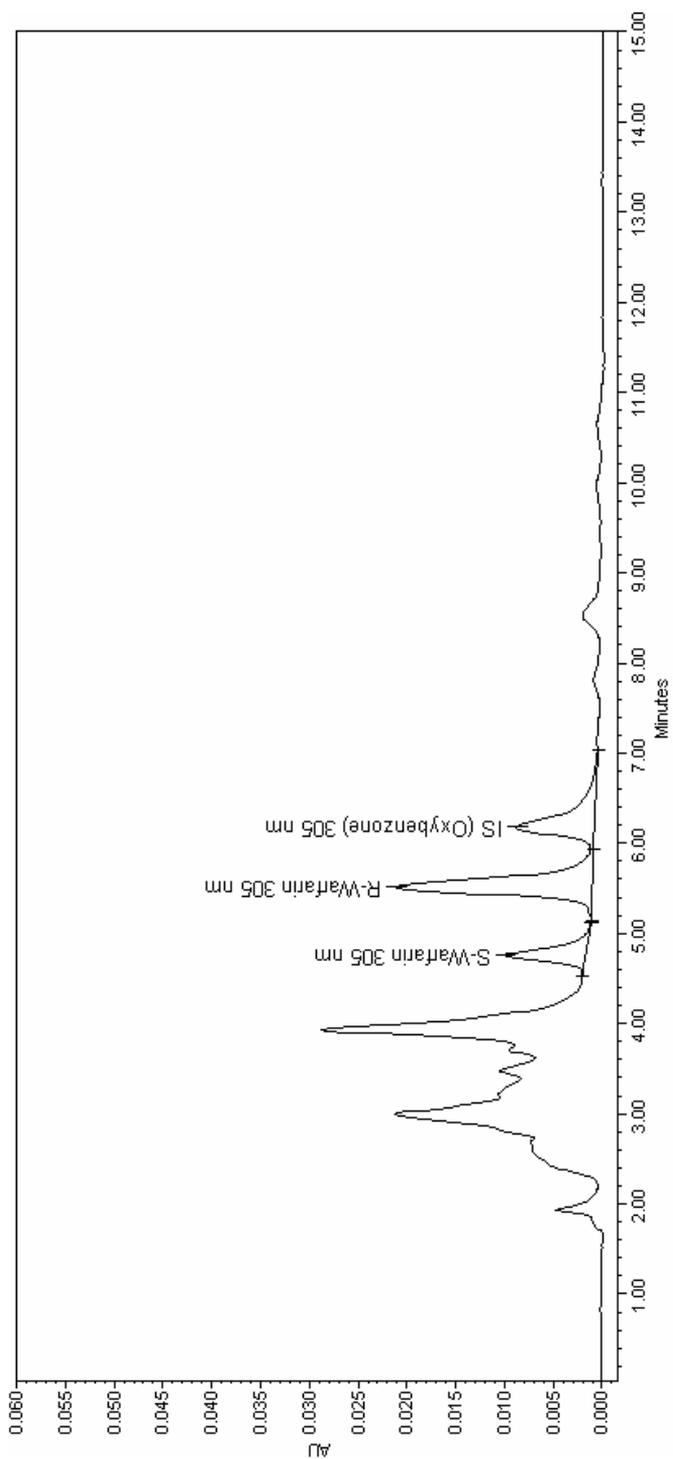


Fig. 13. A chromatogram showing warfarin and IS peaks. The sample was prepared from plasma of an anticoagulated patient and had concentrations of 1.1  $\mu\text{g/mL}$  for (S)-warfarin and 2.4  $\mu\text{g/mL}$  for (R)-warfarin. Retention times (minutes) are on the x-axis while absorbance units (AU) are shown on the y-axis. The mobile phase contained methanol/acetonitrile/water (50/10/40, v/v/v) with 0.1% glacial acetic acid. Detection wavelength was 305 nm with Waters photo diode array detector.

Samples of 251 stable anticoagulated patients were analysed in the study, and the levels of plasma (S)- and (R)-warfarin were investigated (Figure 14). Due to the faster clearance of (S)-warfarin, the mean S/R-warfarin ratio was found to be approximately 0.71 in patients.

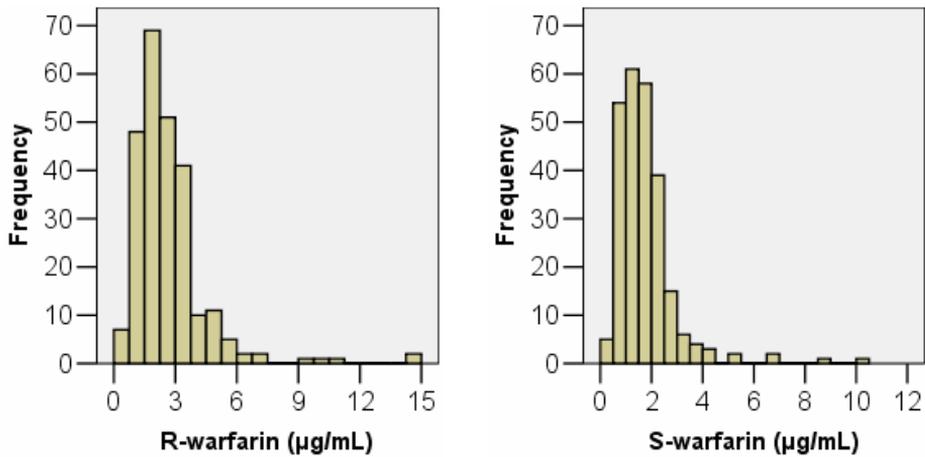


Fig. 14. The distribution of (R)- and (S)- warfarin in 251 anticoagulated patients from south-eastern Sweden. The mean  $\pm$  SD values ( $\mu\text{g/mL}$ ) were  $2.71 \pm 1.84$  for (R)-warfarin, and  $1.74 \pm 1.17$  for (S)-warfarin.

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## Paper II

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In this Paper, the impact of common noncoding *VKORC1* polymorphisms on warfarin treatment was explored. The coding regions and the exon/intron boundaries of the gene were also sequenced for possible mutations in the studied population. Two patients were excluded from the study due to insufficient compliance as warfarin could not be detected in their plasma. An additional four patients could not be analysed for different technical reasons, making the total number of patients who participated in the study 92.

Three SNPs segregating the main haplotypes of *VKORC1* in Caucasians (6484C>T, 9041G>A and 6009C>T) (see Table 2) according to the classification by Geisen et al. [120] were genotyped by DHPLC. The allele frequencies of these SNPs in the studied population are shown in Figure 15. Both patients and controls were exclusively Swedish Caucasians and their allele frequencies were therefore consistent with that of European Caucasians previously reported by others [119,120]. A novel mutation was detected in one patient at nucleotide position 6648 (c.202) involving a C>T transition and consequently a H68Y exchange. This position is in the vicinity of a previously reported mutation (V66M) that was associated with warfarin resistance [118]. Samples from parents of this individual were genotyped (after their informed consent) and the H68Y exchange was found to be of maternal heredity. This patient was also heterozygous for both 9041G>A and 6484C>T SNPs. The H68Y subject had a weekly warfarin dose of nearly 60 mg, and when this was compared with doses for a group of patients with similar *VKORC1* genotype, the H68Y patient was presented as an outlier (see group \*2 + \*4 of Figure 2B in Paper II).

The impact of *VKORC1* common polymorphism on warfarin dose was explored. Only polymorphism 6484C>T demonstrated a clear gene-dose effect and a statistically significant difference in warfarin dose between wild-type (C/C), heterozygous (C/T) and minor allele (T/T) variants. The SNPs 9041G>A and 6009C>T were less predictive than the 6484C>T SNP. To compare the three polymorphisms against each other, only patients homozygous for the minor allele were selected. When warfarin doses of these individuals were compared, a clear and statistically significant difference ( $P < 0.001$ ) was evident between 6484T/T (haplotype *VKORC1*\*2) and 9041A/A or 6009T/T SNPs (*VKORC1*\*3 or *VKORC1*\*4 haplotypes respectively). Patients homozygous for *VKORC1*\*2 required much lower doses than those who were homozygous for *VKORC1*\*3 or *VKORC1*\*4 (Figure 16). There was no significant difference in warfarin dosage between *VKORC1*\*3 and *VKORC1*\*4. The wild-type haplotype, *VKORC1*\*1, was not found in the patient and control populations. Patients with different *VKORC1* haplotypes also showed differences in their plasma warfarin, with *VKORC1*\*2 patients having statistically significant lower plasma (R)-warfarin ( $P < 0.01$ ) than patients with *VKORC1*\*3 or *VKORC1*\*4. The latter two groups had similar (R)-warfarin levels. No differences in plasma (S)-warfarin were obtained between the three groups.

To investigate the impact of *VKORC1* common polymorphism on PT-INR, a retrospective analysis of case records was conducted. This study revealed for the first time that the low dose *VKORC1*\*2 haplotype is associated with difficulties in attaining stable PT-INR. Patients with this variant had higher number of INR values outside the therapeutic range and higher CV of INR in the initiation phase and in the most recent 12 months of treatment than patients with *VKORC1*\*3 or \*4 (Table 4). The clinical relevance of *VKORC1*\*3 and *VKORC1*\*4, on the other hand, seemed to be less obvious. Patients with these two variants had somewhat underestimated doses in their initiation period, but their INR values were stable in the long term compared with patients with *VKORC1*\*2.

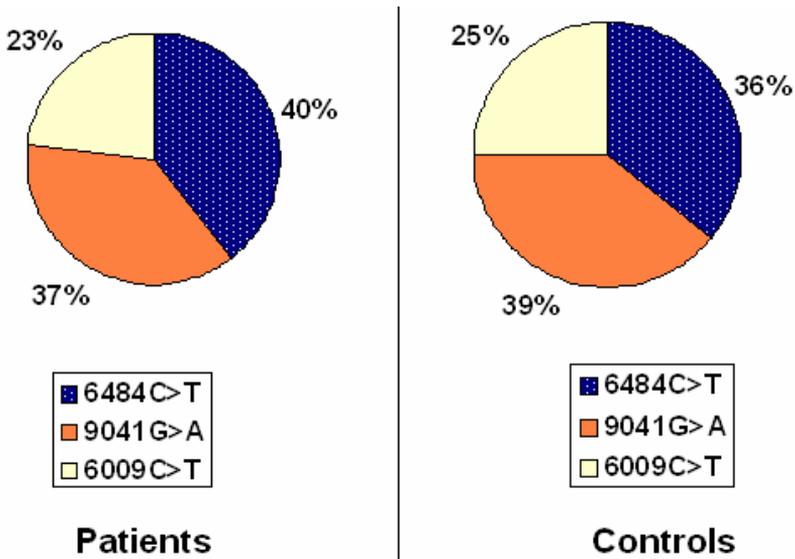


Fig. 15. The three *VKORC1* SNPs investigated in the study and their allele frequencies in 92 patients and 180 healthy controls from the south-eastern region of Sweden.

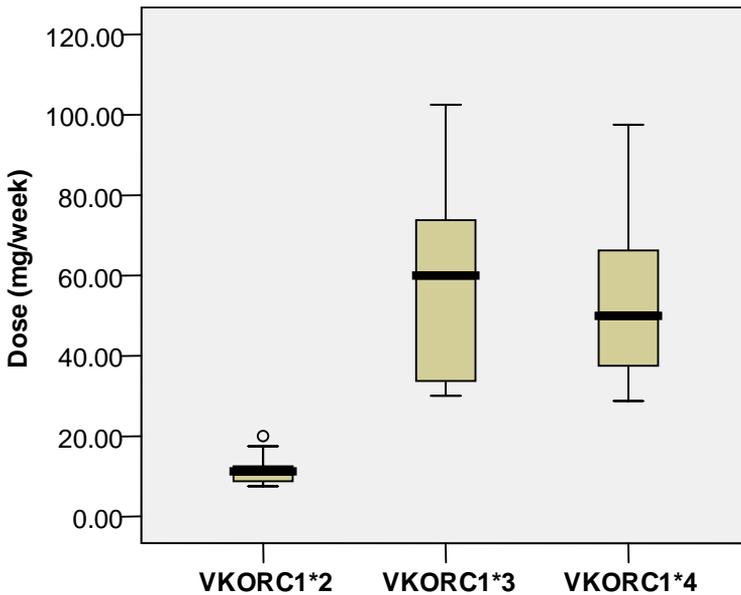


Fig. 16. Inter-quartile range of warfarin dose obtained from patients with *VKORC1*\*2 ( $n = 18$ ), *VKORC1*\*3 ( $n = 16$ ) and *VKORC1*\*4 ( $n = 7$ ) illustrated in Box-and-whisker plot. Each box contains values between 25th and 75th centiles. Horizontal line indicates the median. An individual outlier (o) is also shown.

Table 4.

Retrospective analysis of case records for 10 patients homozygous for *VKORC1*\*2 and 11 patients homozygous for *VKORC1*\*3 or \*4 haplotypes.

-----	VKORC1*2	VKORC1*3 or *4
Number of patients	10	11
Mean age (years)	73.9	73.0
Mean INR in the initiation phase	2.52	2.02
Mean INR in the most recent 12 months	2.40	2.48
Percent INR > 3 in the initiation phase	22.6	2.20
Percent INR < 2 in the initiation phase	18.5	46.2
Percent INR > 3 in the most recent 12 months	10.9	8.0
Percent INR < 2 in the most recent 12 months	16.4	8.0
Mean CV <sup>§</sup> in the initiation phase	32.7	24.0
Mean CV in the most recent 12 months	20.1	12.8
Mean number of visits in the initiation phase	8.90	8.70
Mean number of visits in the most recent 12 months	21.2	11.8

§ = Variation Coefficient

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## Paper III

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This study followed the work presented in Paper II. As mentioned above, a difference in plasma (R)-warfarin levels was observed between patients with *VKORC1*\*2 and those with *VKORC1*\*3 or *VKORC1*\*4. No difference in (S)-warfarin was detected, however, and the question that arose from this observation was whether the S/R ratio of warfarin varies depending on patient's *VKORC1* haplotype and whether this is correlated to *CYP2C9* genotypes. Two patient groups were examined. One group consisted of 17 patients carrying homozygous *VKORC1*\*2 with low warfarin doses. The other group included 22 high-dose individuals who were homozygous for either *VKORC1*\*3 or *VKORC1*\*4 variants. A statistically significant difference in warfarin S/R-ratios was observed between the two groups ( $P < 0.01$ ) (Figure 17). To investigate whether this difference was associated with *CYP2C9* polymorphism, the samples were genotyped for *CYP2C9*\*2 and \*3. However, only three patients with heterozygous *CYP2C9*\*3 were found in the whole patient population (one heterozygous *CYP2C9*\*3 in each *VKORC1* haplotype group). Thus, the difference in warfarin S/R-ratio could not originate from, or be explained by, the most common *CYP2C9* polymorphism. This result is the first indication showing that *VKORC1* haplotypes are associated with warfarin's S/R-ratios.

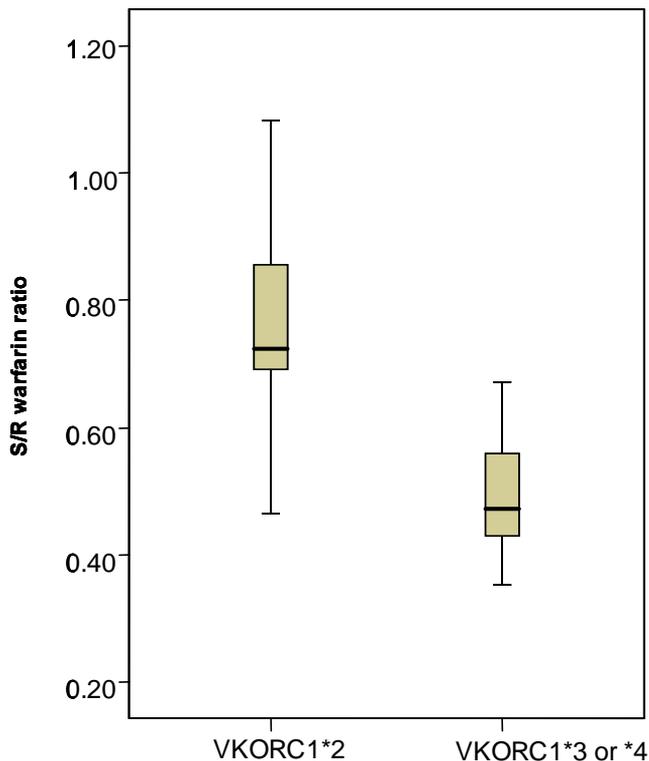


Fig. 17. Warfarin S/R-ratios of 17 patients homozygous for *VKORC1*\*2 and 22 patients homozygous for either *VKORC1*\*3 or \*4. Each box contains values between 25th and 75th centiles. Horizontal line indicates the median.

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## Paper IV

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In this study, possible causes of the variations frequently observed between the different PT methods were investigated. To examine whether the source of depleted plasma influences the relationship between different PT-reagents, bovine and human deficient plasma were compared by preparing two PT-reagents, each containing rabbit thromboplastin but with different depleted plasma (bovine or human). Patient samples ( $n = 40$ ) were then analysed with these reagents. Figure 18 shows the result from this study. It was demonstrated that the source of deficient plasma does not contribute to between-reagent variance. Good correlation was found between the two reagents ( $r = 0.99$ ,  $y = 1.20x + 1.40$ , at  $P < 0.001$ ).

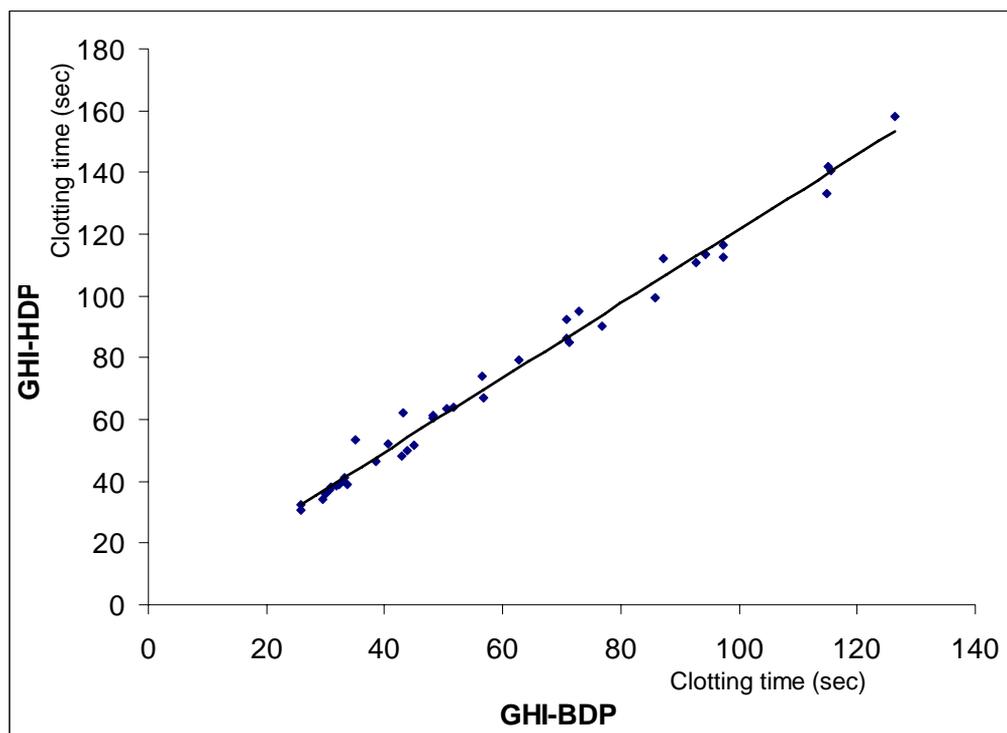


Fig. 18. The relationship between two rabbit thromboplastin (GHI) reagents containing human depleted plasma (HDP) or bovine depleted plasma (BDP) used to analyse 40 anticoagulated patients.

To study the effect of sample dilution on between-reagent variations, patient samples ( $n = 34$ ) were analysed undiluted,  $\times 2$ ,  $\times 7$ ,  $\times 10$  and  $\times 12$  diluted, with two different reagents containing either Thromborel-S thromboplastin (prepared from human placenta) or GHI thromboplastin (prepared from rabbit brain). Both reagents were prepared as Owren reagents and contained bovine deficient plasma as a source of fibrinogen and factor V. Calcium was held constant at optimal concentration. The results revealed that the predilution of the sample is an important factor, playing a critical role in the correlation between different reagents. The poorest correlation ( $r = 0.67$ ) between GHI and Thromborel-S reagents was found when the sample

was undiluted. The best correlation was obtained at 7-fold dilution ( $r = 0.95$ ). Further sample dilutions did not improve the correlation. Figure 19 shows the relationship between the two reagents for undiluted (Quick style) and 7-fold (Owren style) sample dilutions. All correlation coefficients were significant at  $P < 0.001$ . In Figure 20, the correlation coefficients and dilutions are plotted against each other.

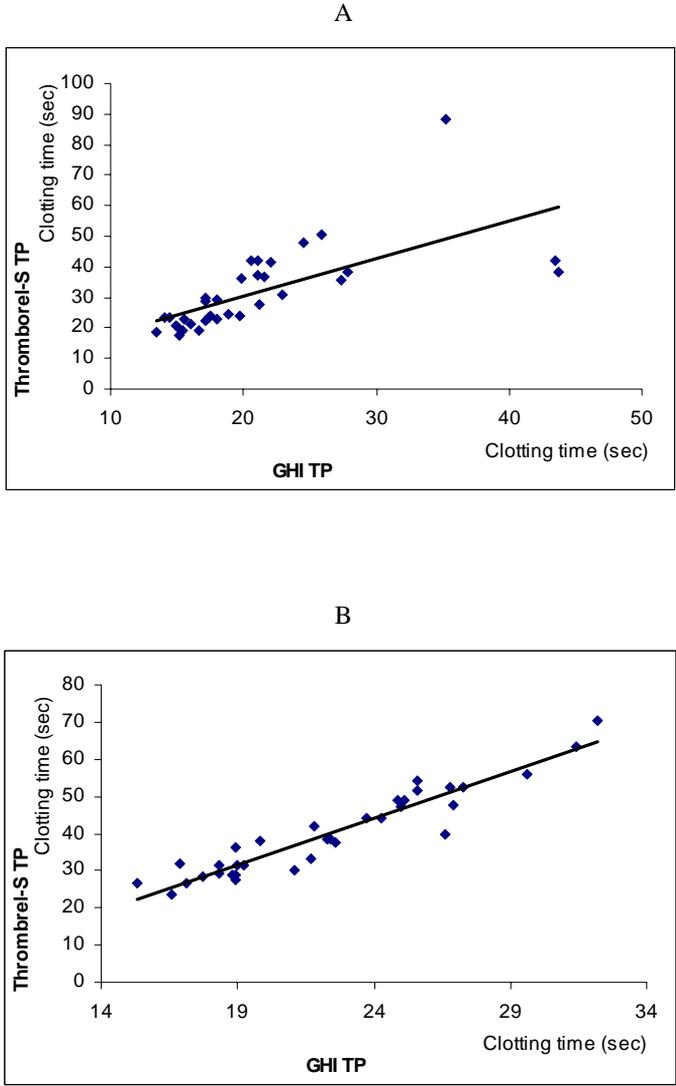


Fig. 19. The relationship between GHI and Thromborel-S thromboplastin (TP)-reagents for undiluted (A) and 7-fold prediluted (B) samples ( $n = 34$ ).

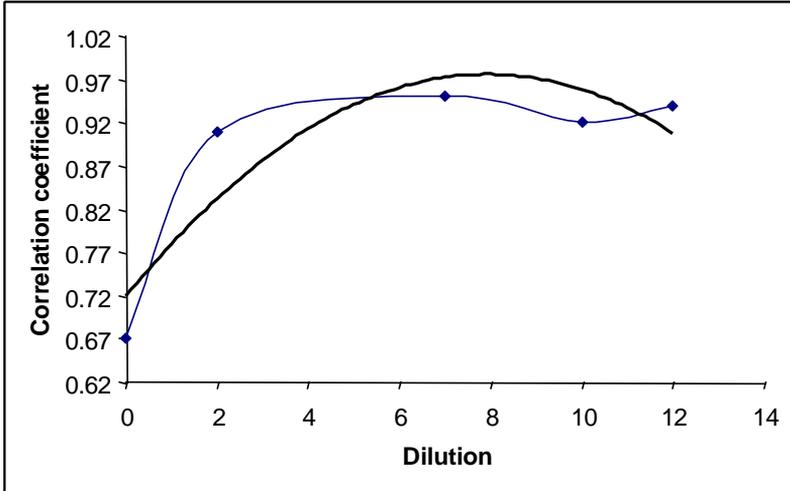


Fig. 20. A graph showing the relationship between correlation coefficients and sample dilutions when rabbit (GHI) and human (Thromborel-S) thromboplastin reagents were compared. A line connecting the actual points are shown. The other line is obtained when a polynomial cubic fitting is performed according to the equation:  $y = b + c_1x + c_2x^2 + c_3x^3 + \dots + c_6x^6$ .

# DISCUSSION

The anticoagulant effect of warfarin is monitored by analysis of prothrombin time expressed as INR. However, measurement of plasma warfarin is necessary in some situations where information is required on the metabolism and clearance profile of the drug, or on patient compliance. In this thesis, a chiral HPLC method for measuring plasma warfarin enantiomers was developed (Paper I). A number of different HPLC methods for the determination of plasma warfarin have been identified in the literature (Table 3). Many of these methods were either too laborious or they could not be reproduced at our laboratory. The chiral columns used to resolve drugs are expensive and sometimes not durable. Expected cost, including materials and time, is therefore high if the method is not reasonably stable and reproducible. A cyclodextrin column was initially tested according to the method of Naidong et al. [130]. This column, however, often lost activity even though the recommendation of the manufacturer was carefully followed. A Pirkle (R,R) Whelk-01 column was therefore installed due to its recognised stability and robustness. With this column, a good chromatographic resolution and sufficient sensitivity was obtained. The column was furthermore sustainable for several hundreds of injections with no sign of reduction in activity or resolution. All patient samples contained amounts of warfarin well above the LOD of the method. The sample with the lowest concentration of warfarin contained amounts which were more than 20 times above LOD and more than 2 times above the limit for quantitation.

The relationship between PT-INR and plasma warfarin was studied. There were no significant correlations between PT-INR and S-warfarin or between PT-INR and (R)-warfarin. This confirms the general observation showing the lack of correlation between prescribed warfarin dose and clinical effect. To attain a therapeutic INR, which is usually 2 – 3, the dose of warfarin is individualised. Within this therapeutic range, the dose for two given patients can differ dramatically.

The relationship between (S)- and (R)-warfarin was also investigated. The (S)-enantiomer has approximately 2 – 5 times faster clearance than the (R)-isomer, and the question was whether the two enantiomers were correlated in the studied population. In Figure 21, the relation of (S)-warfarin to its (R)-counterpart is plotted for 251 anticoagulated patients. There was a significant but not a perfect correlation between the two optical isomers ( $r = 0.82$ ,  $y = 0.5236x + 0.33$ ). The correlation coefficient was not close to 1.00, and this can be explained by possible variations of warfarin clearance in the studied population. The phase I metabolism of the more potent (S)-warfarin in humans is well understood. In contrast, the bioconversion of (R)-warfarin and the number of enzymes involved in the metabolism of this isomer have not yet been fully clarified.

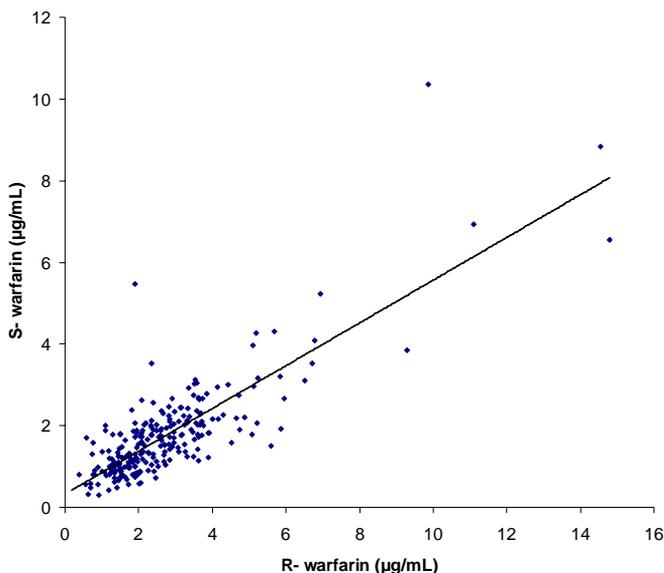


Fig. 21. A diagram showing the relationship between (S)- and (R)-warfarin in 251 anticoagulated patients. All patients in the study had stable INR-values for a period of time and were assumed to have steady-state plasma warfarin.

In Paper II, common *VKORC1* polymorphisms influencing warfarin treatment were studied. Although many reports have demonstrated the impact of these SNPs on warfarin dose requirement, our study has shown for the first time that the low dose *VKORC1*\*2 haplotype is also clinically significant for the stability of PT-INR. A small retrospective analysis of patient records revealed that patients homozygous for this haplotype had significantly more fluctuations in their INR than those who were homozygous for *VKORC1*\*3 or *VKORC1*\*4 ( $P < 0.01$ ). Patients with *VKORC1*\*2 had 36% and 57% more variations of PT-INR in their initiation phase and in their most recent 12 months, respectively, than patients with *VKORC1*\*3 or \*4. *VKORC1*\*2 patients also displayed more INR values outside the therapeutic range, which is associated with increased risk of adverse events. However, the number of samples involved in the study was too small (22 patients) to draw any general conclusion or to recommend DNA testing for all patients prior to initiation of anticoagulant therapy. Further studies are needed to confirm these results. To elucidate, for instance, whether *VKORC1*\*2 patients visit anticoagulation clinics more often than the *VKORC1*\*3 or \*4 patients during long-term warfarin treatment, a larger retrospective or prospective study would be needed. With a power of 85%, a standardised difference of 0.67 (calculated from our study) and a significance level of 0.05, such a study should typically contain 80 patients in each group.

In Paper III, the relation between *VKORC1* polymorphism and warfarin S/R-ratio was studied. It is important at this point to mention that altered warfarin S/R-ratio has generally been used as an indicator of changed *CYP2C9* activity [133,151]. In this study, however, a statistically significant difference in warfarin S/R-ratios was observed between patients with *VKORC1*\*2 and those with *VKORC1*\*3 or \*4, but this difference was not related to the *CYP2C9* genotype. This result is interesting and has not previously been reported. It challenges the general

assumption that any change to warfarin S/R-ratio has to do with an alteration of *CYP2C9* enzymatic function.

The SNPs in *CYP2C9* and *VKORC1* genes are inherited independently and the observed change of warfarin S/R-ratio in this study is therefore distinct from other S/R alterations associated with *CYP2C9* polymorphisms. The S/R ratio of warfarin is variable in patient populations as can be seen in Figure 22 for 251 anticoagulated individuals regardless their *CYP2C9* or *VKORC1* polymorphisms (mean  $\pm$  SD = 0.71  $\pm$  0.34). Interestingly, the variation coefficient (CV) of S/R-ratios for each *VKORC1* patient group (CV = 31 for *VKORC1*\*2 and CV = 24 for *VKORC1*\*3 or \*4 patients) was lower than the CV for the general population (CV = 48), although the general population was larger than the two groups investigated in the study. The mechanisms linking warfarin S/R-ratios and *VKORC1* haplotypes are at present not known. Patients with *VKORC1*\*2 require generally much lower warfarin doses than other patients, as described in Paper II. It is possible that this difference in doses might be associated with dose-dependent variations in clearance rates of warfarin enantiomers. It is also conceivable that *VKORC1* SNPs are in linkage disequilibrium with other polymorphisms involved in the metabolism or elimination of warfarin.

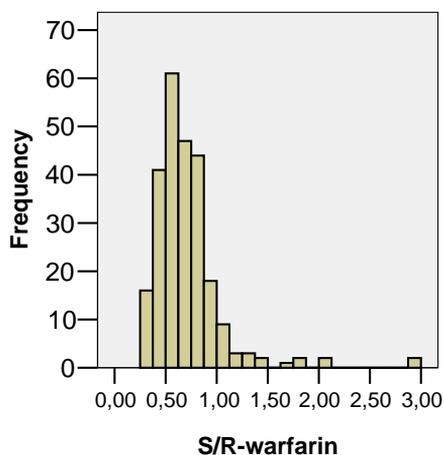


Fig. 22. Plasma warfarin S/R-ratios for 251 anticoagulated patients.

The influence of SNPs on the *VKORC1* gene function have drawn attention in the last three years, and knowledge on these genetic markers and their interaction with oral anticoagulant therapy is growing. The importance of *VKORC1* polymorphism for other conditions than warfarin treatment has also been highlighted. Wang et al. [152], for instance, recently found that *VKORC1*\*2 haplotype is a genetic marker for coronary heart diseases and stroke. The same haplotype has been found to be involved in the altered correlation between the concentration of unbound (S)-warfarin and INR [123]. This haplotype is also related to a lower mRNA transcription of *VKORC1* [119] and an altered binding site motif in *VKORC1* for nuclear factor 1 (NF1) [125]. Overall, the studies in Papers II and III have confirmed some of the previous observations regarding the importance of *VKORC1*\*2 and added new knowledge demonstrating that *VKORC1*\*2 may be clinically significant for warfarin treatment and is associated with altered S/R-ratio of warfarin. The other haplotype combinations in *VKORC1* have not shown any clinical relevance thus far.

In Paper IV, the effect of dilution on the measurement of PT-INR was investigated. It is interesting that the original 1-stage method of Quick's from the mid 1930s is still in use in almost unchanged form. At the time Armand Quick developed his PT test, the classical four-factor theory was still valid, and prothrombin was the only known coagulation factor apart from fibrinogen, tissue factor and calcium. Luckily, prothrombin happened to be the most important coagulation factor in haemostasis and has a longer half-time in plasma than the other vitamin K-dependent coagulation factors (VII, IX and X), which were discovered after the Quick PT method. The current Quick methods used worldwide are principally not different from the original test by Quick. One part of undiluted sample is mixed with two parts of a reagent containing equal parts of thromboplastin and calcium. On the other hand, a greater variety of different thromboplastins with varying sensitivities is now available in the market than at the time Quick developed his test, which is not just an advantage as they contribute to the inter-laboratory variations seen in Quick methods.

Despite many international efforts and the introduction of INR calibration, Quick methods are still associated with large inter-laboratory variations not observed in the countries where Owren's method is used. Paul Owren recognised already in the 1950s the advantage of sample dilution, which obviously reduces the effect of different inhibitors. Moreover, the addition of deficient plasma containing sufficient amounts of factor V and fibrinogen makes the Owren method more specific for the factors repressed by warfarin than do Quick methods.

Paper IV shows that better agreement in PT results can be achieved for Quick methods if the sample is prediluted as in the Owren method. The two reagents compared in the study had the best correlation when the sample was 7× diluted (Owren style) ( $r = 0.95$ ). In contrast, undiluted samples (Quick-style) presented the poorest correlation between the two reagents ( $r = 0.67$ ). It seems, therefore, that some of the inter-laboratory variations in Quick methods are originated from a lack of sample predilution. In the Nordic countries where the Owren method is applied, much better precision is obtained in the external quality control programmes. If laboratories changed from Quick methods to Owren methods the quality of control of anticoagulation would most likely improve and benefit millions of patients.

# CONCLUDING REMARKS

The studies presented in this thesis have extended the current knowledge on anticoagulant therapy in the following ways:

- » A simple and reproducible HPLC method for the measurement of warfarin enantiomers has been developed and validated. With this method, the levels of plasma warfarin following its oral administration can be studied and evaluated. Abnormal clearance in some patients can be detected, and patient compliance can be verified. Furthermore, differing ratios of (S)- and (R)-isomers can be identified. The current method is robust and compared to other published methods simple and cost-effective.
- » The impact of *VKORC1* haplotypes on warfarin dose requirement, plasma levels of warfarin and the stability of PT-INR in patients was clarified. In the study, *VKORC1*\*2 haplotype has been identified as an important genetic determinant of warfarin dosage. Patients with this variant had also significantly lower plasma levels of (R)-warfarin. Furthermore, this haplotype was associated with difficulties not only in attaining but also in retaining stable therapeutic PT-INR. Patients with *VKORC1*\*2 had higher fluctuations of their INR in the initiation phase and in the most recent 12 months of their treatment, and more INR values outside the therapeutic range than patients with other *VKORC1* haplotypes.
- » Significant difference in S/R ratio of plasma warfarin was found between patients carrying different *VKORC1* haplotypes. This difference could not be attributed to the *CYP2C9* genotype, indicating a possible interplay between warfarin's enantiomeric ratio and *VKORC1* enzyme activity.
- » The causes of poor agreement between PT methods were explored. This study has shown that sample dilution according to the Owren method used in the Nordic countries is to be preferred for the harmonisation of PT results. In contrast, undiluted samples according to the Quick methods, used in most countries, had presented insufficient correlation when two different thromboplastin reagents were compared.

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# APPENDIX

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## Theory and terminology of high-performance liquid chromatography (HPLC)

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The term chromatography comes from the Greek words *chroma* (colour) and *graphein* (write). It was in use already in the 19<sup>th</sup> century in arts and colours [153]. More than 100 years ago, the Russian botanist M. S. Tswett was studying the physiochemical nature of plant pigments. In 1903 he reported a method which he had used to separate chlorophyll molecules using a column filled with chalk ( $\text{CaCO}_3$ ) (in [154]). Tswett called his method *chromatography*, and his invention became the starting point of the modern analytical chemistry. In a simplified manner, chromatography is a technique used to separate a mixture of analytes (i.e. the substances to be analysed) by distributing them between two phases. Usually, one phase is stationary and the other is mobile. When the stationary phase is solid and the mobile phase is liquid, the term LC (liquid chromatography) is used. Different types of LC techniques are generally classified based on the type of chromatographic distribution (or equilibrium) [155], such as ion-exchange, size-exclusion, gel-permeation, and adsorption chromatography. The latter is the most frequently used LC form and is subdivided into *normal* phase (the stationary phase is more polar than the mobile phase), *reversed* phase (the mobile phase is more polar than the stationary phase), *affinity* (an interaction between an immobilised ligand and molecules that selectively bind to it), and chiral LC (stereo-specific separation of enantiomers).

An LC technique utilising a high-pressurised system, with a pulse-free pump continuously delivering the mobile phase, is called HPLC (high-performance or high-pressure LC). HPLC is a powerful tool developed in the 60's and 70's and is used for the separation of substances with varying properties, such as indigenous and food molecules, drugs, industrial organic substances, and biopolymers. The power of HPLC arises from its flexibility, with almost boundless combinations of stationary and mobile phases. In contrast to gas chromatography, HPLC does not require vaporisation of the substances to be analysed, and can be analysed with molecules up to several million Daltons [155]. A typical HPLC system contains a pump that passes a flow of liquid (mobile phase) through an injector from where the sample is applied under pressure. The sample molecules are transported into the column, where an interaction with the sorbent particles takes place. Finally, the analytes pass through a detector that sends a signal to a computer or integrator for peak integration and reporting.

The distribution of an analyte between the stationary and the mobile phases is thermodynamically described by the following equation [156]:

$$K_c = \frac{C_s}{C_m} \times \frac{V_s}{V_m} \quad \text{Eq. 1}$$

Where:

$K_c$  = the distribution constant,  $C_s$  = molar concentration in the stationary phase,  $C_m$  = molar concentration in the mobile phase,  $V_s$  = volume of the stationary phase and  $V_m$  = volume of the mobile phase

A component that has no interaction with the stationary phase moves down the column at the speed of the mobile phase and has an elution time of  $t_0$  ("dead time"). On the other hand, a component that spends a part of its time on the stationary phase and part of its time in the mobile phase is retained in the chromatographic system with a retention time of  $t_R$ . Such analytes are said to have a chromatographic *capacity* ( $k'$ ). The magnitude of  $k'$  largely depends on the nature of the analyte, the temperature, and, most importantly, on the strength of the mobile phase and the chemical property of the stationary phase. The capacity factor is calculated according to the following equation:

$$K' = \frac{t_R - t_0}{t_0} \quad \text{Eq. 2}$$

The ability of a chromatographic system to retain a solute stronger or weaker than another is called the *selectivity* ( $\alpha$ ). The  $\alpha$  factor (also called *the separation factor*) is dependent on the chemical properties of the analyte as well as on the mobile and the stationary phase, and is described by the following equation:

$$\alpha = \frac{K'_2}{K'_1} \quad \text{Eq. 3}$$

Where  $k'_1$  and  $k'_2$  represent the capacity factors of components 1 and 2.

An important chromatographic property is the column *efficiency* ( $N$ ), which describes the band spreading of the samples. Chromatographic peak shapes ideally follow a Gaussian distribution. Diffusion and mass transfer effects, however, cause the bands to spread as they move through the column packing. The more the components diffuse through the stationary particles, the broader the Gaussian shape they will have. A column with good efficiency has a high number of plates (segments) and therefore a sharper Gaussian-style peaks. The following equation describes the column efficiency:

$$N = 16 \left( \frac{t_R}{W_b} \right)^2 \quad \text{Eq. 4}$$

Where  $t_R$  is the retention time and  $W_b$  is the tangential peak width.

Another chromatographic term is *resolution* ( $R_s$ ), which describes how two peaks (or components) are separated from one another.

$$R_s = \left(\frac{1}{4}\right) \left(\frac{K'}{K'+1}\right) \left(\sqrt{N}\right) \left(\frac{\alpha-1}{\alpha}\right) \quad \text{Eq. 5}$$

As stated in equation 5, the resolution of a chromatographic system is determined by column efficiency and selectivity as well as the capacity factors. Whereas column efficiency is mainly dependent on the particle size of the stationary phase, and the length and internal diameter of the column,  $\alpha$  and  $k'$  factors are defined by many other factors including sample preparation, composition and strength of the mobile phase, type of detection, and the nature of the stationary phase.

To identify the analytes leaving the outlet of the column, and to study their separation characteristics, an HPLC detector is required. In the early years of HPLC development, refractive index (RI) and conductivity detectors were widely used. But the sensitivity of these instruments was insufficient and the introduction of inline UV and fluorescence detectors was a major achievement for HPLC development. UV-absorption detectors respond to those substances that absorb light in the range 180 to 350 nm. Initially, fixed wavelength UV detectors, with a mercury vapour lamp that generated most of its light at a wavelength of 254 nm, were used. Although the sensitivity was nearly a thousand times better than that of RI detectors, not all samples have absorbance at 254 nm and the use of single-wavelength detectors became limited to only few substances [155]. It was therefore a major improvement when variable wavelength UV detectors were introduced into the market in the late 1970s.

A type of more useful UV detector is the photo diode array (PDA). PDA consists of a number of photosensitive diodes placed on one another in the form of multi-layer sandwich. The diodes receive a polychromatic light from a lamp. By diffraction grating on to the surface of diode array, each diode receives a light of different wavelength. This way, an absorbance spectrum can be obtained containing data from many wavelengths.

Some form of UV-absorbance detector accounts for probably 80% of all HPLC detection (<http://www.chromatography-online.org/>). Other common techniques include fluorescence, electrochemical, and in recent years mass spectrometric detections.

### ***Internal standard calibration***

In HPLC, as in other analytical chemistry, calibration of the analysis is often performed by using an internal reference component called internal standard (IS). The standard curve and the unknown samples to be analysed, are spiked with IS in equal amounts. The IS substance should neither be an indigenous substance nor a foreign compound found in the sample matrix. It is added to the samples at the early stages of sample preparations. The advantage of IS calibration is that any possible errors in dilutions will be the same for both IS and sample components. To calculate the concentration of an unknown sample, standard curves are generated by putting the concentrations of the standards on the  $x$  axis and the response ratios on the  $y$  axis. Response ratio is defined as the signal ratio between the calibration standard and the IS multiplied by the concentration of the internal standard.

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