Two Worlds, One Goal: A Clinician’s Perspective on Laboratory Analyses in Anticoagulant Treatment

Kerstin Arbring
Overview of the coagulation system (secondary hemostasis)

Intrinsic/Contact pathway
Activation by negatively charged surfaces

Extrinsic/Tissue factor pathway
Vessel damage exposing TF

Overview of coagulation factors:
- **Prothrombin (II)** (Vitamin K-dependent)
- **Thrombin (IIa)**
- **Fibrinogen (I)**
- **Fibrin (Ia)**
- **Fibrin (crosslinked)**
- **Fibrin degradation products**
- **Protein S**
- **Protein C**
- **APC**
- **EPCR**
- **TAFI**
- **TFPI**
- **AT**

Activation and Inhibition:
- Activation
- Inhibition

Adapted from figure provided with the courtesy of PhD Kenny Hansson.
Two worlds, one goal:
A Clinician’s Perspective on Laboratory Analyses in Anticoagulant Treatment

Kerstin Arbring

Division of Diagnostics and Specialist Medicine
Department of Health, Medicine and Caring Sciences
Faculty of Medicine and Health Sciences
Linköping University, Sweden
Linköping 2023
To my family,
two- and fourlegged,
with love

“Being a little weird
is just a natural side-effect
of being awesome”

Sue Fitzmaurice
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>POPULÄRVETENSKAPLIG SAMMANFATTNING</td>
<td>3</td>
</tr>
<tr>
<td>PAPERS INCLUDED IN THIS THESIS</td>
<td>5</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>7</td>
</tr>
<tr>
<td>AIMS</td>
<td>9</td>
</tr>
<tr>
<td>PREFACE</td>
<td>11</td>
</tr>
<tr>
<td>1. HEMOSTASIS — walking the tightrope</td>
<td>13</td>
</tr>
<tr>
<td>1.1 Introduction to hemostasis</td>
<td>14</td>
</tr>
<tr>
<td>1.2 Overview of hemostasis</td>
<td>15</td>
</tr>
<tr>
<td>1.3 Examples of hemostatic disorders</td>
<td>18</td>
</tr>
<tr>
<td>2. ORAL ANTICOAGULANT DRUGS—sweet clover disease heritage and beyond</td>
<td>21</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>22</td>
</tr>
<tr>
<td>2.2 Warfarin — the story of a cattle tragedy</td>
<td>23</td>
</tr>
<tr>
<td>2.3 Warfarin, VKORC1, and the vitamin K cycle</td>
<td>24</td>
</tr>
<tr>
<td>2.4 Warfarin and genetic polymorphisms</td>
<td>26</td>
</tr>
<tr>
<td>2.5 Warfarin — challenges in treatment</td>
<td>27</td>
</tr>
<tr>
<td>2.6 DOACs (or NOACS?—no, not anymore) — a paradigm shift</td>
<td>30</td>
</tr>
<tr>
<td>3. THE LABORATORY CHALLENGE—understanding from bits and pieces.</td>
<td>35</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>36</td>
</tr>
<tr>
<td>3.2 Prothrombin Time, still going strong</td>
<td>36</td>
</tr>
<tr>
<td>3.3 PT-INR meets DOACs</td>
<td>40</td>
</tr>
<tr>
<td>4. CONTRIBUTIONS OF THIS THESIS—towards towards the goal</td>
<td>41</td>
</tr>
<tr>
<td>4.1 Introduction and Overview</td>
<td>42</td>
</tr>
<tr>
<td>4.2 Paper I: The VKORC1 gene and its influence on warfarin treatment</td>
<td>43</td>
</tr>
<tr>
<td>4.3 Paper II: On organizing VKA treatment— primary health care centers or centralized anticoagulation clinics?</td>
<td>45</td>
</tr>
<tr>
<td>4.4 Paper III: A new prothrombin time method as a tool for measuring DOACs</td>
<td>48</td>
</tr>
<tr>
<td>4.5 Paper IV: Avoiding erroneous laboratory results caused by DOAC interferences with coagulation analyses</td>
<td>50</td>
</tr>
<tr>
<td>4.6 Ethical considerations</td>
<td>51</td>
</tr>
<tr>
<td>5. CONCLUDING REMARKS</td>
<td>53</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>57</td>
</tr>
<tr>
<td>References</td>
<td>63</td>
</tr>
<tr>
<td>Paper I-IV</td>
<td>76</td>
</tr>
</tbody>
</table>
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT
ABSTRACT

Almost precisely a century ago, in the 1920s and 1930s, cattle bled to death in North America after being fed moldy hay containing sweet clover, the yellow *Melilotus officinalis*, and the white *Melilotus albus*. The toxic substance in the hay inhibiting blood coagulation was identified and named dicumarol. Further development resulted in warfarin, an oral anticoagulant that has been used for over 70 years and still is, even though newer direct-acting oral anticoagulants (DOACs) are mainly replacing it. For some patients, warfarin is still the drug of choice. A safe warfarin treatment needs repeated blood sample analysis (PT-INR), and with the new DOACs come new laboratory challenges. The aim of this thesis was to investigate ways laboratory methods can contribute to improving oral anticoagulant treatment.

Paper I explores genetic variants of the enzyme targeted by warfarin, VKORC1. The result shows that the haplotype VKORC1*2 is the most important of the VKORC1 haplotypes for warfarin dosage, with a lower dose requirement. The VKORC1*2 haplotype was also related to more unstable PT-INR levels.

Paper II describes a cross-section study comparing warfarin treatment control, as PT-INRs within the intended therapeutic range, in primary health care centers (PHCCs) and specialized anticoagulation clinics (ACCs). Both settings showed good therapeutic control, with at least as good therapeutic control in the PHCCs as in the ACCs. Today, almost all warfarin treatment in our region is centralized to ACCs.

Paper III focuses on the modification of a point-of-care PT method. A ratio of PT from two different dilutions of each patient sample was calculated and used as an indirect measure of DOAC activity. There were close correlations between the PT ratio and drug concentrations measured at the hospital laboratory. The detection level varies between DOACs and may limit its use in some situations.

Paper IV evaluated the MRX PT DOAC, an assay based on the PT ratio principle. It was found to be able to detect potentially interfering DOAC levels in plasma samples. Confirmatory testing is recommended, as is sensitivity improvement for the detection of specific interferences.
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT
SAMMANFATTNING

POPULÄRVETENSKAPLIG SAMMANFATTNING


I delarbete I har vi sett att det finns en koppling mellan vissa genetiska varianter och ett lägre behov av warfarin, och likaså en mer svårinställd behandling.

I delarbete II undersökte vi kvaliteten på warfarinbehandlingen i primärvård jämfört med specialiserade antikoagulantia(AK-)mottagningar genom jämförelse av hur PK-proven under en viss vecka förhöll sig till önskade värden. Båda vårdformerna visade god kvalitet på warfarinbehandlingen, minst lika bra i primärvården som på AK-mottagningarna. Sedan studien gjordes har nästan all warfarinbehandling centraliserats till AK-mottagningar.

I delarbete III modifierade vi en analysmetod som vanligen används för analys av PK-prov, med sikte på att också kunna använda den för analys av effekt av de nya antikoagulantialäkemedlen. Vi såg en god överensstämmelse mellan den modifierade metodens resultat och ordinarie analys.

I delarbete IV utvärderade vi MRX PT DOAC, en analysmetod som bygger på den princip som beskrivs i delarbete III. Vi använde MRX PT DOAC för att upptäcka prover med läkemedel som kan störa andra analyser. Vi såg goda resultat men också förbättringspotential.
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT
PAPERS INCLUDED IN THIS THESIS

Paper I


Paper II

Arbring K, Uppugunduri S, Lindahl TL. Comparison of prothrombin time (INR) results and main characteristics of patients on warfarin treatment in primary health care centers and anticoagulation clinics. BMC Health Serv Res. 2013;13(1):85.

Paper III


Paper IV

Arbring K, Lund M, Onelöv I, Lindahl T. Evaluation of MRX PT DOAC as a new screening method for detecting interferences in thrombophilia analyses. (Submitted)

Papers reprinted with permission from the publisher (Paper I), and under Creative Commons license CC-BY https://creativecommons.org/licenses/by/4.0/
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Anticoagulation Clinic</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial Fibrillation</td>
</tr>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CYP(450)</td>
<td>Cytochrome P(450)</td>
</tr>
<tr>
<td>DOAC</td>
<td>Direct Oral AntiCoagulant</td>
</tr>
<tr>
<td>DTI</td>
<td>Direct Thrombin Inhibitor</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep Vein Thrombosis</td>
</tr>
<tr>
<td>DXI</td>
<td>Direct (Factor) X(a) Inhibitor</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-carboxyl glutamic acid</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>INR</td>
<td>International Normalized Ratio</td>
</tr>
<tr>
<td>IRP</td>
<td>International Reference Preparation</td>
</tr>
<tr>
<td>ISI</td>
<td>International Sensitivity Index</td>
</tr>
<tr>
<td>LA</td>
<td>Lupus Anticoagulant(s)</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low Molecular Weight Heparin</td>
</tr>
<tr>
<td>NOAC</td>
<td>New Oral AntiCoagulant</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasmin Activator Inhibitor-1</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary Embolism</td>
</tr>
<tr>
<td>PHCC</td>
<td>Primary Health Care Center</td>
</tr>
<tr>
<td>POC</td>
<td>Point Of Care</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin Time</td>
</tr>
<tr>
<td>PT-INR</td>
<td>Prothrombin Time expressed as INR</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Controlled Trial</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin Activatable Fibrinolysis Inhibitor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>TR</td>
<td>Therapeutic Range</td>
</tr>
<tr>
<td>TIR</td>
<td>Time In therapeutic) Range</td>
</tr>
<tr>
<td>TTR</td>
<td>Time in Therapeutic Range</td>
</tr>
<tr>
<td>VKA</td>
<td>Vitamin K Antagonist</td>
</tr>
<tr>
<td>VKORC</td>
<td>Vitamin K epOxide Reductase Complex subunit 1</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous ThromboEmbolism</td>
</tr>
<tr>
<td>vWD</td>
<td>von Willebrand Disease</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>α2AP</td>
<td>α2AntiPlasmin</td>
</tr>
</tbody>
</table>
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT
AIMS

Overall aim
To investigate ways laboratory methods can contribute to improving oral anticoagulant treatment.

Specific aims

- 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. (Paper I)

- To compare patient characteristics and PT-INR levels in a cross-section of material from Swedish primary health care centers and specialized anticoagulation clinics. (Paper II)

- To develop a method based on a point-of-care, Owren-type prothrombin time method to measure the effects of direct oral anticoagulants (DOACs) on the extrinsic coagulation pathway. (Paper III)

- To evaluate the ability of MRX PT DOAC, a prothrombin time quotient method, to accurately identify interfering apixaban or rivaroxaban concentrations, non-interfering or interfering patient samples, and whether a patient is on DOAC treatment. (Paper IV)
THISTHESISWILLTAKEYOUONAHEMOSTASIS-THEMEDJOURNEYBETWEENTOWORLDs.
IMAGINEYOUAREAPHYSICIANWITHPATIENTSTREATEDWITHANTICOAGULANTsinana
anything-but-clear-cutorpreciseclinicalpracticeworld(orifthatispreciselywhat
andwhereyouare,thenjustfeelit!).Seeyourselflookingthroughthewindowofthelaboratoryworld,fascinatedbyalltheprecisearswersproducedthere,seeingthемagiciansturningbloodinfoactsandfiguresbywavingtheirwands.Now,youfind
adoor(anda passkey!),andbeforeyoudo,knowityouhaveentered.Themomentthedoorclosesbehindyou,yourealizeyouhavenomap(andoifyouareanythinglikeme,
youimmediatelyfeeltheneedtostartdroppingbreadcrumbs to have any chance at
alloffindingyourwayback).Butnowneedtoworry!

Thepeople dressed in lab coats (some may be quietly hiding behind the big lab
instruments) are kind magicians! They will speak gently to you, show you around,
offeryouacupofcoffee,makeyoufeelwelcometoday,andeveryoubackthenextday.Theywillforgiveyoufornotunderstanding,andtheywillhavethepatience
toexplain,overandoveragain,inthehopeyouwillunderstandsomeday(andoftenyou
do,andthenforgetagainafterawhile,theywillصارalloveragain).Gettingtoknowyou,they will start asking you about your world, and you will be slightly
bewildered at how genuinely interested they are — why?

Andthenoneday,probablyat fika-time,youwill suddenly realize why:throughtheir
window,lookingintoyourworld,theyseemagiciansmakingpatientsbetter,even
curingthem,bywavingclinicianwands.Fromthattodayforward,youwillstrivetomake
theseworlds understand each other better. You see the need to set up tours
between the worlds, encouraging magicians of all sorts to come along in both
directions. Because when they speak the same language and know the strengths and
weaknesses of one another and one another’s wands, the magicians of the two
worlds can truly unite their forces to improve patient care — including, but not
limited to, anticoagulant treatment.

Now, grab your wands, and let’s embark on this journey!
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT
1. HEMOSTASIS – walking the tightrope

“Not all those who wander are lost.”

Bilbo Baggins
in The Lord of the Rings
by J.R.R. Tolkien
1.1 Introduction to hemostasis

Hemostasis is the intricate balance between bleeding and thrombosis. Not enough, and potentially life-threatening bleeding might be imminent; too much, and a clot might detach and result in an equally life-threatening embolus. This fine-tuned defense system includes blood vessels, platelets, and the proteins called coagulation factors.

Although still referred to as “primary hemostasis”, comprising the initial role of vessels and platelets, and “secondary hemostasis”, with the role of the coagulation factors in mind, current knowledge recognizes a cell-surface, interplay model where the two are tightly intertwined and to a large extent simultaneous (Hoffman & Monroe, 2001). With that in mind, and without contradicting it, an overall understanding of an essential system in the context of anticoagulants and laboratory analyses can be reached in more than one way.

The coagulation system is ancient, sharing ancestry and general layout with the complement system — another critical defense system in vertebrates (Dzik, 2019; Noris & Galbusera, 2023). They both have cascade-like enhancement systems along main roads with reinforcement loops to support them, self-regulating mechanisms preventing overshoot, and interplay in health and disease (Dzik, 2019; Noris & Galbusera, 2023). Innumerable clinicians have been terrified by the mere sight of the “coagulation cascade” (as well as that of the complement system). Unfortunately, many do not understand what a useful general understanding they miss out on if they run in fear of overwhelming details. The following aims to give a short overview of hemostasis, from resting platelet to stabilized clot, without causing too many casualties from drowning in details. (Tip: On the inside of the front cover of this book, there is a map!)
1.2 Overview of hemostasis

After an initial spasm following a vessel injury, the very first line of defense, the platelets, are the vanguard of the hemostatic forces (Scridon, 2022). As an effect of blood rheology, the small platelets are constantly pushed toward the vessel walls. This proximity is beneficial when a vessel is injured. The von Willebrand factor acts as a catchline anchored in the exposed subendothelial tissue (J. E. Sadler, 1998). Platelet rolling is initiated, followed by adhesion, activation, granular release, and aggregation to form a platelet plug (Scridon, 2022).

Without a stabilizing fibrin net, the platelet plugs will most likely succumb to, and be swept away by, the blood flow. Coagulation factors, a protein family of Roman-numbered proenzymes, will come to its rescue in the coagulation part of hemostasis. The coagulation factors circulate mainly in their inactive form in plasma. The complete nomenclature for these zymogens is a capital F followed by the Roman number for the factor, as in FVIII or FX. When they are activated through proteolytic cleavage, turning into functional enzymes, an “a” is added to their name. Although all coagulation factors also have a specific name, just a few names are in common use: Fibrinogen (FI), Fibrin (FIa), prothrombin (FII), and thrombin (FIIa).

In vivo, the combination of tissue factor (TF) released from the injured tissue and FVII (FVIIa circulating in low concentrations in plasma) is the main coagulation initiator. The first coagulation phase, the Initiation phase (Hoffman & Monroe, 2001), occurs on the endothelial cell surface. The complex of TF+FVIIa (and calcium) converts FX to FXa and FIX to FIXa, but only in small amounts in this phase. Likewise, at most, just small amounts of thrombin are produced. The next phase, the Amplification phase, requires the phospholipid surface provided by the activated, shape-changed platelets (Hoffman & Monroe, 2001; Scridon, 2022). Here, the conditions are suitable for the coagulation factors to assemble and the two main enzyme complexes to form: the “tenase” complex, consisting of FIXa with its cofactor FVIIIa (and calcium), activating FX into FXa, and the “prothrombinase” complex, with FXa joining forces with cofactor FVa (and calcium), activating prothrombin into thrombin.
When these two complexes are established, the two most crucial intersections for boosting coagulation are up and running — activation of FX and prothrombin, respectively. The production of thrombin increases into a “thrombin burst”, and the final thrombin-producing phase is reached, the *Propagation phase* (Hoffman & Monroe, 2001). The thrombin cleaves fibrinogen into fibrin and accelerates its own production by adding to the activation of FXI, FVIII, and FV, the latter two both serving as cofactors in the tenase and prothrombinase complexes, respectively.

The activations of FXI, FVIII, and FV happen along a second line of activation. The first line of activation, starting with FVIIa+TF, has been called the “extrinsic pathway”; the second, the “intrinsic pathway,” starts by the activation of FXII. At the level of FXa, the lines are joined into the “common path”. The alternative naming of the two initial lines, “tissue factor pathway” and “contact pathway”, is somewhat closer to the truth as the latter was found to be activated in vitro by contact with e.g., glass surfaces. In vivo, if there are no artificial surfaces in contact with the plasma, the first part of the contact pathway is less important for initiating hemostasis.

Down the line of the common path, thrombin also activates the last of the Roman-numbered factors, FXIII, into FXIIIa. FXIIIa contributes to the crosslinking of fibrin, a prerequisite for the robust fibrin mesh needed to stabilize the platelet plug and protect it from degradation.

Observing the positive feedback from thrombin, it is possible to identify its similarity with the complement system — one main road (TF+FVII, via its activation of FX into FXa, down to thrombin and fibrin) with an attached reinforcement loop (thrombin feedback and then down again via the tenase and prothrombinase complexes).

Thrombin has a wide diversity of roles to play, both pro- and anticoagulant (Al-Amer, 2022; Lane et al., 2005). In addition to the activation of coagulation factors, thrombin contributes to platelet activation at the injury site. The way it turns from being pro- to anticoagulant is a key to the necessary self-regulation of the system. The two most important anticoagulants are antithrombin and Protein C. Antithrombin contributes primarily to the inactivation of thrombin and FXa. Protein C is activated into activated Protein C (APC), which, potentiated by its cofactor
Protein S, accelerates the degradation of FVIIIa and FVa. Thrombin activates Protein C when bound to thrombomodulin. Thrombin encounters thrombomodulin on endothelial cells where the endothelium is intact, thus protecting the non-damaged endothelium from being clotted. Likewise, thrombin binds to heparan sulfate on intact endothelium and is then inactivated by antithrombin (Al-Amer, 2022; Lane et al., 2005; Olson, 2002).

The last part to be mentioned is the fibrinolysis, the “cleaning squad” that degrades the mesh on the hemostatic plug, eventually leaving the repaired vessel wall free. The critical player in fibrinolysis is plasmin. Plasminogen is primarily activated into plasmin by tissue plasminogen activator (tPA) (Risman et al., 2023; Sillen & Declerck, 2021). Fibrinolysis is also subject to a fine-tuned regulation: tPA is inhibited by plasmin activator inhibitor-1 (PAI-1) and plasmin deactivated by α2antiplasmin (α2AP). Also, thrombin and plasmin activate thrombin activatable fibrinolysis inhibitor (TAFI), which hinders plasminogen activation of the fibrin surface (Risman et al., 2023; Sillen & Declerck, 2021).
1.3 Examples of hemostatic disorders

There are numerous steps in the hemostasis process where a slight change in structure or function can lead to imbalance. Just a few of them will be mentioned here.

**Primary hemostasis**

Moderate to severe bleeding symptoms are seen in Bernard-Soulier syndrome due to reduced expression of the GPIb platelet receptor (Andrews & Berndt, 2013; López et al., 1998), and in Glanzman thrombasthenia due to reduced expression of the GPIIbIIIa platelet receptor (Franchini et al., 2010). von Willebrand disease (vWD) leads to mild to severe bleeding symptoms due to a quantitative or qualitative deficiency in von Willebrand factor; in more severe cases also secondary hemostasis with a decreased level of FVIII as the von Willebrand factor acts as its carrier and protector (B. Sadler et al., 2022; Vangenechten & Gadisseur, 2022).

**Secondary hemostasis**

The best-known hereditary factor deficiencies are hemophilia A (reduced level of FVIII) and hemophilia B (reduced level of factor IX), both of which are X-linked and can lead to bleeding symptoms ranging from mild to severe (Castaman & Matino, 2019). Notably, both of the affected factors can be found in the “reinforcement loop” of the coagulation cascade. Deficiency in FIX, sometimes called hemophilia C, is autosomally inherited and is associated with more variable bleeding symptoms (Lewandowska & Connors, 2021). Further up in the “intrinsic pathway,” FXII factor deficiencies do not lead to bleeding symptoms.

FVII deficiency is probably the most common factor deficiency. It has a very high variation of bleeding symptoms, from none to severe, without an apparent correlation with the factor level and with varying prevalence in different cohorts (Robinson, 2019; Trillo et al., 2022).

On the other hand, deficiencies in the anticoagulant proteins (antithrombin, protein C, or protein S) lead to a thrombotic tendency, which can result in venous
thromboembolism (VTE), i.e. deep venous thrombosis and pulmonary embolism (Khan & Dickerman, 2006). Also, a mutation in FV, FV Leiden, leads to FV being more resistant to degradation by APC and is the most common cause of APC resistance, increasing the risk of thrombosis (Khan & Dickerman, 2006; Morimont et al., 2022). Mutations in the prothrombin gene can also result in thrombotic tendency (Khan & Dickerman, 2006).

Much more can be written about hereditary bleeding and thrombotic disorders, but that would need another book.
2. ORAL ANTICOAGULANT DRUGS—sweet clover
disease heritage and beyond

“They never ceased to wonder,
they kept on trying,
and they were on a project directed toward
doing mankind some good instead of trying
to destroy it.”

Karl Paul Link (1959, p. 106)
2.1 Introduction

An antithrombotic drug reduces hemostasis somewhere along the line. There are manifold reasons why a patient would benefit from an antithrombotic drug. Indications can be primary or secondary prevention of clotting. Common causes include prevention of myocardial infarction, stroke prevention in atrial fibrillation, prevention of postoperative thrombotic complications, and treatment of venous thromboembolism (VTE), as in deep vein thrombosis (DVT) and pulmonary embolism (PE). Different diagnoses require different types of antithrombotic treatment. Generally, arterial clotting diseases (as prevention of clotting in coronary arteries) need drugs targeting the primary hemostasis, and venous clotting diseases (as in VTE) need drugs targeting the secondary hemostasis due to different flow and pressure conditions in the respective vessel systems. (But no rule without exception: stroke prevention in atrial fibrillation warrants drugs targeting the secondary hemostasis because of “venous-like flow conditions” in the heart’s left atrium.)

Drugs targeting the primary hemostasis are commonly called “platelet inhibitors”, targeting platelet enzymes or receptors; aspirin, a nonspecific cyclo-oxygenase COX) inhibitor, clopidogrel, and ticagrelor, P2Y12-receptor antagonists (irreversible and reversible respectively) are well-known representatives.

In this thesis, the focus is on managing anticoagulants, i.e., drugs targeting hemostasis at the coagulation factor level (the secondary hemostasis), and especially those that can be administered orally; warfarin, a well-known so-called vitamin K antagonist, and the newer, direct oral anticoagulants apixaban, dabigatran, edoxaban, and rivaroxaban.

Parenteral anticoagulants are not the main focus but need mentioning; heparin, nowadays almost exclusively administered intravenously, and low molecular weight heparin (LMWH), fractions of heparin for subcutaneous administration, and fondaparinux, a synthetic pentasaccharide with similar action as the LMWH.
2.2 Warfarin — the story of a cattle tragedy

A tragedy that took place a century ago eventually led to the discovery of oral anticoagulants. In the 1920s and 1930s, cattle in North America bled to death in what came to be known as sweet clover disease. This disease was brought on by the cattle being fed moldy hay containing sweet clover, the yellow *Melilotus officinalis*, and the white *Melilotus albus* (Link, 1959; Schofield, 1924, 1984). The disastrous effect on the cattle consuming this hay was suggested in 1929 to result from a prothrombin reduction, causing prolonged coagulation time (Roderick, 1931).

The toxic agent in the hay causing this was later (1939) isolated, characterized (and subsequently synthesized), and given the name dicumarol (Campbell et al., 1940; Campbell & Link, 1941; Stahmann et al., 1941). Among various analogs synthesized by Link's group over the next few years, one was highly potent and initially used as rat poison. This substance was named warfarin, an abbreviation of Wisconsin Alumni Research Foundation coumarIN (Link, 1959). Human treatment with dicumarol was introduced in the 1940s (Lehmann, 1959) and with sodium warfarin at the beginning of the 1950s (Link, 1959; Mannucci & Poller, 2001).

![Molecular structures of warfarin and dicumarol](image)

**Figure 1.** The molecular structures of warfarin and dicumarol. The asymmetry of the warfarin molecule is the ground for chirality (center marked with *). *Figure and partial legend provided through the courtesy of Dr. Abdimajid Osman (2007, p. 5).*
2.3 Warfarin, VKORC1, and the vitamin K cycle

Coumarins lead to an anticoagulant effect by acting as vitamin K antagonists (VKAs). Vitamin K-dependent FII, FVII, FIX, and FX (and the likewise vitamin K-dependent Protein C and Protein S) all need γ-carboxylation to acquire the capability to bind calcium, which is a prerequisite for them to be functional. This γ-carboxylation requires the reduced form of vitamin K. Vitamin K epOxide Reductase Complex subunit 1 (VKORC1) is responsible for reducing vitamin K from its oxidized form. The provision of vitamin K can function as a VKA antidote (L Poller, 1996; Link, 1959).

Figure 2. The vitamin K cycle. In step 1, vitamin K quinone (K) is reduced to vitamin K quinol (K\(\text{H}_2\)) by vitamin K reductase (VKR) or possibly by vitamin K epoxide reductase (VKOR). In step 2, γ-glutamyl carboxylase uses K\(\text{H}_2\), oxygen, and carbon dioxide to convert glutamic acid (Glu) residues on the vitamin K-dependent protein into γ-carboxyl glutamic acid (Gla). As a reaction by-product, vitamin K epoxide is formed which must be reduced back to K by VKOR, a warfarin-sensitive enzyme (step 3). Figure and legend provided through the courtesy of Dr. Abdimajid Osman (2007, p. 17).
Warfarin is administered as a racemic mixture of these R- and S-enantiomers, both of which are highly protein-bound in plasma with only the free fraction pharmacologically active (O’Reilly, 1969, 1974; Wittkowsky, 2003).

The two enantiomers differ in potency, the S-enantiomer being 2–5 times more potent than R-warfarin but with a shorter half-life, range 18–52 h for S-warfarin and 20–70 h for R-warfarin (O’Reilly, 1974; Wittkowsky, 2003). This can be compared to the half-life of the hemostasis-related Vitamin K-dependent proteins (Table 1) (Wittkowsky, 2003).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FII</td>
<td>42–72</td>
</tr>
<tr>
<td>FVII</td>
<td>4–6</td>
</tr>
<tr>
<td>FIX</td>
<td>21–30</td>
</tr>
<tr>
<td>FX</td>
<td>27–48</td>
</tr>
<tr>
<td>Protein C</td>
<td>9</td>
</tr>
<tr>
<td>Protein S</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1. Half-life of vitamin K-dependent, coagulation related, proteins (Wittkowsky, 2003)
S-warfarin is considered to be the clinically most relevant enantiomer for the anticoagulant response to warfarin treatment, but R-warfarin is notably dominant in plasma at steady state due to its longer half-life (Takahashi & Echizen, 2001; Wittkowsky, 2003).

The enantiomers are metabolized in the liver by different cytochrome P450 (CYP) enzymes: S-warfarin primarily via CYP2C9; R-warfarin partially via CY1A2, CYP3A4 and CYP2C19 (Wittkowsky, 2003). The CYP metabolism exposes warfarin to numerous interactions with drugs and dietary supplements (Wittkowsky, 2001, 2003, 2008).

### 2.4 Warfarin and genetic polymorphisms

The genetics of the pharmacodynamic site of warfarin, the VKORC1, was explored later than polymorphisms in CYP2C9 alleles related to the pharmacokinetics of (S-)warfarin. Alleles CYP2C9*2 and CYP2C9*3 are related to a reduced dose requirement (Takahashi & Echizen, 2001). In a Swedish population, the allele frequencies were CYP2C9*1 81.9%, CYP2C9*2 10.7%, and CYP2C9*3 7.4% (Yasar, 1999).

Single nucleotide polymorphisms (SNPs) in the VKORC1 gene, mainly in non-coding regions, have been related to warfarin dose requirement, with significant differences described between populations in the distribution of different haplotypes (Geisen et al., 2005; Rieder et al., 2005; Takahashi et al., 2006):

VKORC1*1 (ancestral/wild type) together with VKORC1*3 dominates in Africans, whereas VKORC1*2 dominates, with up to 90% prevalence, in Asian populations; VKORC1*2 and VKORC1*3 dominate in Europeans. In Paper I, we explored this in a Swedish setting, relating haplotypes to warfarin concentrations in plasma and warfarin dose requirements.
2.5 Warfarin — challenges in treatment

The coumarins/VKAs have been extensively used for many years, with warfarin during its heyday being the most prescribed anticoagulant worldwide. The VKAs have been used for primary and secondary prevention and treatment for arterial and venous thrombosis, e.g., in patients with atrial fibrillation, venous thrombosis, and prosthetic heart valves. Even though the VKAs are now overshadowed by newer anticoagulants in many of their former indications, they are still in use. As an example, in the last of the patient groups mentioned above, patients with mechanical heart prostheses, warfarin is still the drug of choice for the prevention of clotting and embolizing (Eikelboom & Weitz, 2023; Wang et al., 2023).

VKA treatment is well known to lead to challenges due to a narrow therapeutic range. Under-anticoagulation may lead to thrombotic complications as manifestations of the underlying condition, events the VKA treatment is supposed to prevent. Over-anticoagulation, on the other hand, increases the risk of severe or even fatal bleeding. Reported frequencies of VKA treatment complications vary widely. Major bleeds have retrospectively been described to occur in more than 2% of VKA-treated patients with mechanical heart valves (Cannegieter et al., 1995), but numbers vary widely.

Bleedings requiring hospital care in VKA-treated patients have previously been reported in 1.7–16% of which 0.3–7% were fatal (L Poller, 1996). In random controlled trials (RCTs) for the new oral anticoagulants in atrial fibrillation (see below), the numbers for yearly incidences of major hemorrhage and intracranial bleeding in the warfarin groups were approximately 3.0–3.5% and 0.4–0.85%, respectively (Connolly et al., 2009; Giugliano et al., 2013; Granger et al., 2011; Patel et al., 2011). The differences in bleeding rates may be explained by several factors like different treatment indications or intensities, age of patients, concomitant medication, propensity to report, and treatment organization and control.
Good therapeutic control of the VKA treatment can keep adverse events (bleeding or thrombosis) to a minimum (Ansell et al., 2008; White et al., 2007). To achieve this, treatment must be monitored by repeated analysis of prothrombin time (PT, expressed as International Normalized Ratio, INR) because of the large variation in dosing required for optimal INR. Both inter- and intraindividual differences cause the wide variation in weekly dose (up to twentyfold). The metabolism of VKA drugs differs substantially between individuals.

Genetic differences are appreciated to account for approximately 40% of the inter-individual variation in initial warfarin response and clinical factors for just above 10% in addition to that, leaving almost half of the variation unaccounted for (Li et al., 2019). The relation between genetic differences in pharmacodynamic response to warfarin and INR within the intended therapeutic range is addressed in Paper I. The intra-individual variation over time is caused by e.g., variations in concomitant medication or variable ingestion of vitamin K with food (Geisen et al., 2005).

VKA treatment control is traditionally measured as time in therapeutic range (TTR), or just time in range (TIR), referring to the proportion of time a patient spends having an INR within the intended therapeutic range—the higher, the better. TTR is usually calculated as described by Rosendaal et al. (1993) and expressed as a percentage of time. Sweden is among the very best at keeping VKA-treated patients within their therapeutic range, which is often evident in RCTs when exposed, with Sweden having a TTR of close to 80% (Connolly et al., 2008; Wallentin et al., 2010, 2013). In Figure 4, TTR is illustrated for anticoagulation clinics in our region.
How VKA treatment is organized differs worldwide depending on available facilities and opinions concerning the best way to do it. Sweden has had some counties with predominantly specialized anticoagulation clinics (ACCs), usually in hospitals, whereas others predominantly managed treatment at the patient’s health care center (PHCCs).

Over recent decades, specialized anticoagulation clinics have come to dominate. As this trend started even before the newer oral anticoagulants made an entrance, the grounds for this shift are somewhat unclear and probably extend beyond strictly medical reasons. Both regimes coexisted earlier in our region, and in Paper II, we began to address the question of treatment quality in different settings. Close in time to that, our region decided to centralize all warfarin dosing to ACCs for mainly organizational reasons, as I understand it.
Regardless of the reason for the decisions about the organization, the situation has fundamentally changed. Currently, the predominance of newer drugs brings new challenges: how can the art of warfarin dosing be maintained to serve patients who still need their warfarin treatment? And do the newer drugs need monitoring in clinical practice? These questions will not be answered in this thesis, but ensure that there will be more to do tomorrow.
2.6 DOACs (or NOACS?—no, not anymore)—a paradigm shift

In September 2009, the results from the RE-LY study were published, comparing an oral (direct) thrombin inhibitor (DTI), dabigatran etexilate, brand name Pradaxa®, with warfarin in the prevention of stroke in atrial fibrillation. The results showed at least non-inferiority in effect and bleeding risk, with a significantly lower incidence of hemorrhagic stroke (Connolly et al., 2009). This marked the beginning of a new era in oral anticoagulant treatment; in late 2011, the first patient in Sweden was prescribed dabigatran.

Thrombin and FXa are both products of the two main enzyme complexes underlying the “thrombin burst,” as described earlier. While dabigatran targets thrombin, in the following few years results from studies on three oral (direct) FXa inhibitors (DXI) were published, all initially on stroke prevention in atrial fibrillation:

- **ARISTOTLE**, apixaban (brand name Eliquis®), September 2011 (Granger et al., 2011)
- **ROCKET AF**, rivaroxaban (brand name Xarelto®), September 2011 (Patel et al., 2011)
- **ENGAGE AF-TIMI 48**, edoxaban (brand name Lixiana® in Sweden), November 2013 (Giugliano et al., 2013)

Studies on VTE treatment with these new drugs, including dabigatran, followed:

- **RE-COVER**, dabigatran, September 2009 (Schulman et al., 2009)
- **EINSTEIN-DVT** and **EINSTEIN-PE**, rivaroxaban, December 2010 (Bauersachs et al., 2010) and April 2012 (Büller HR et al., 2012), respectively
- **AMPLIFY-EXT** and **AMPLIFY**, apixaban, February 2013 (Agnelli et al., 2012) and August 2013 (Agnelli et al., 2013)
- **Hokusai-VTE**, edoxaban, September 2013 (Hokusai-VTE Investigators, 2013)
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT

Oral anticoagulants targeting FXI are being tested in ongoing phase 3 trials (abelacimab, asundexian, and mivexian), and those targeting FXII are reported to be under development (Fredenburgh & Weitz, 2023). But for now, the DTI and DXIs described above, are the DOACs available on the Swedish market.

Apixaban was the only one of the drugs that showed overall superiority to warfarin in these RCTs (Granger et al., 2011). All studies showed a lower incidence of hemorrhagic stroke, which has since been the hallmark of all these DOACs, aside from their dosing regime. Apixaban was the only drug with an overall lower incidence of major gastrointestinal bleeding in the RCTs (different results for different doses with dabigatran and edoxaban). In essence, real-world data seems to concur with the bleeding profiles from the RCTs (Ballestri et al., 2023).

When the results from these DOAC studies reached Sweden, they led to discussions on the applicability of the results in the Swedish setting because it was noticed that the overall TTRs in these studies were surprisingly low. Moreover, these are well-controlled studies, ranging from 55% (Patel et al., 2011) to just above 68% (Hokusai-VTE Investigators, 2013). Of course, it is hard to compare groups between studies, and the patient characteristics differed somewhat, but the questions were legitimate.

A clarifying detour on the name of these drugs: Initially, the new drugs were collectively called just that, new oral anticoagulants (NOAC). Realizing there will always be new drugs emerging, and today’s new drugs will be tomorrow’s old ones, it was suggested that the abbreviation NOAC should stand for non-vitamin K antagonist oral anticoagulants instead. Finally (?) a new denominator was suggested, direct oral anticoagulant (DOAC), referring to their mode of action (and defining them for what they are, not what they are not). The transition from NOACs to DOACs was the quickest in settings where the drug itself was in focus, with its pharmacological and laboratory effects, but today (October 2023), DOAC gives far more hits in a PubMed search than NOAC does. The denominator DOAC will be used in this text, but NOAC was used in Paper III, in line with its time of publishing.
When this is written, in October 2023, DOACs are well-established in both atrial fibrillation and venous thromboembolism. For patients with mechanical heart valves, this is not the case. Another trial was recently stopped due to thrombotic events in the DOAC (apixaban) group (Eikelboom & Weitz, 2023; Wang et al., 2023). Experience of DOAC patients with severely impaired kidney function remains limited, and caution is recommended for the more severely ill patients with antiphospholipid syndrome (APS), as described in a recently published comprehensive review (Olie et al., 2024).

In Sweden there has been a definite switch from predominantly VKA treatment to the DOACs, predominantly the DXIs, see Figure 5 for an overview (both sexes combined, same trend for both, not shown here). Currently just below 170,000 patients are treated with oral anticoagulants, approx. 1.6% of the population, with a marked dominance from the DXI. There are regional differences in preferences though, see Figure 6 (and please observe the different scales for the coloring!).

**Figure 5.** Anticoagulant use over time. Data (2006-2022) was retrieved from the Swedish National Board of Health and Welfare, data processing, and visualisation were done in R (Neuwirth, 2022; R Core Team, 2023; Wickham et al., 2019)
Figure 6 a) VKA (vitamin K antagonist) treatment in Sweden

Figure 6 b) DTI (thrombin inhibitor) treatment in Sweden

Figure 6 c) DXI (FXa inhibitors; rivaroxaban, apixaban, edoxaban) treatment in Sweden

Figure 6. Regional distribution of oral anticoagulant use in Sweden. Data (for September 2023) was retrieved from the Swedish National Board of Health and Welfare, data processing, and visualization were done in R ver 4.3.1 (Hijmans et al., 2023; R Core Team, 2023; Wickham et al., 2019) also including the sf package.
3. THE LABORATORY CHALLENGE—understanding from bits and pieces.

“Oh Lord, may I be directed what to do and what to leave undone”

Elisabeth Fry
3.1 Introduction

This section is an overview of the laboratory interface. The PT method is described to facilitate understanding of Paper III and Paper IV. As for the genes in Paper I, the technical details are beyond the scope of this thesis (and me, I am not a chemist!). The focus is on the background of our research questions and the clinical interface.

Using laboratory methods to understand a whole picture is like doing a jigsaw puzzle. The picture is there when all the bits and pieces are in place. Until then, the bits can be a bit confusing.

3.2 Prothrombin Time, still going strong

Under-anticoagulation puts the patient at risk of thrombotic events, whereas over-anticoagulation increases the risk of bleeding. Both can be equally threatening to the patient’s well-being, or even life. For safe treatment, we need to titrate the VKA dosage over time as the dose-response relationship shows a wide inter- and intra-individual variation, making it hard to predict the appropriate dosage. Such titration requires us to be able to measure the anticoagulant effect of the VKA drug with a reliable, well-standardized test.

Prothrombin time, PT, is still the laboratory method of choice to measure the anticoagulant effect of VKAs. The original prothrombin time test, the Quick PT, was designed by Armand Quick in the mid-1930s (Quick, 1935; Quick et al., 1935):

\[
\text{Prothrombin} + \text{thromboplasin} + \text{calcium} \rightarrow \text{thrombin} \\
\text{Fibrinogen} + \text{trombin} \rightarrow \text{fibrin}
\]
And for thromboplastin:

“The traditional term “thromboplastin” refers to a phospholipid-protein extract of tissue (usually lung, brain, or placenta) that contains both the tissue factor and phospholipid necessary to promote activation of factor X by factor VII. Thromboplastins vary in responsiveness to the anticoagulant effects of warfarin according to their source, phospholipid content, and preparation.” (Hirsh et al., 2003, p. 1636)

Today we are blessed with the possibility to use recombinant human thromboplastin.

The prothrombin content was related to the time it took to form a clot in the mixture. Two more vitamin K-dependent factors, FVII and FX, were later discovered to influence the Quick PT. The last of the pro-coagulant vitamin K-dependent factors, FIX, does not influence the test, which can be explained by the in vitro activation caused by the thromboplastin being so strong that the reinforcement loop of the coagulation cascade, where FIX is at work, is not needed to form the clot.

The levels of FV and fibrinogen also influence the Quick PT. This is a disadvantage for monitoring the VKA effect as (only) the drug affects the vitamin K-dependent factors. Fibrinogen, for example, is not influenced by the VKA but can vary with a number of unrelated conditions and may influence the test result, making it less reliable. The Quick PT used today has been modified since Armand Quick first described it, but the same coagulation factors influence the test. The final sample dilution is also still 1+2 (1:3). It is worth noticing that the Quick PT principle is used in the Coagu-Chek® point-of-care equipment. Still, modifications have made it less influenced by fibrinogen (and hematocrit) levels (Wieloch et al., 2009).
In the midst of World War II, the Norwegian physician Paul Arnor Owren treated a spontaneously bleeding patient with a prolonged PT that was corrected by prothrombin-depleted plasma, leading to his discovery of FV (Blombäck, 2001; Douglas, 1999). A few years later, he was also among the first to discover FVII (Blombäck, 2001; Douglas, 1999). In the 1950s, he described a more specific test for the anticoagulant effect of VKA on FII, FVII, and FX (Owren, 1959; Owren & Aas, 1951). In this assay, the blood sample is prediluted in a buffer in proportions 1+6 (1:7). One part of this diluted sample is then mixed with two parts of a reagent containing adsorbed bovine plasma (depleted of the vitamin K-dependent coagulation factors), thromboplastin, phospholipids, and calcium. The test is supplied with FV and fibrinogen from the plasma (but no vitamin K-dependent factors), rendering it sensitive to the factors that interest a VKA-treated patient. The final sample dilution 1+20 (1:21) also reduces exposure to interference factors, e.g., the lupus anticoagulant (as the process highly dilutes these factors).

To ensure a safe VKA treatment based on PT test results, the test has to have a low intra- and interlaboratory variation. When an international normalized ratio (INR) for PT results was recommended by the World Health Organisation (WHO) in 1983, it was an important milestone on the way to a well-needed worldwide standardization (“WHO Expert Committee on Biological Standardization Thirty-Third Report,” 1983).

Using the equation

\[
\text{INR} = \left( \frac{\text{PT}_{\text{patient}}}{\text{PT}_{\text{mean normal plasma}}} \right)^{\text{ISI}}
\]

the ISI, as the international sensitivity index of the thromboplastin, attempts to standardize the effect of the different thromboplastins used. The ISI for a thromboplastin is given by its calibration against the WHO international reference preparation (IRP).
The Owren PT test, to our knowledge currently used in laboratories in the Nordic and Baltic Countries and to some extent, in the Netherlands, is more easily standardized. The use of serial dilutions of normal plasma and a more simplified calculation of INR (without the need for IRP) has been described for the Owren-PT test results (Lindahl et al., 2004), using the equation

$$\text{INR} = \frac{\left( \frac{1}{\text{PT}\%_{\text{Owren}}} + 0.018 \right)}{0.028}$$

The simplified calculation has been proven robust in Swedish laboratories (Hillarp et al., 2004). In addition, the Owren method has some important practical advantages to the Quick method, e.g., the smaller volume of blood required makes capillary blood sampling more feasible, and the independence from the labile FV results in a much better stability of the blood sample.
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT

3.3 PT-INR meets DOACs

With the arrival of DOACs, it was said they needed no testing. Or, since they are more or less dependent on kidney function for their elimination, at least not any testing apart from kidney function. But, there is a clinical reality as well. In this reality, patients bleed sometimes, and DOAC patients as well. And sometimes they have to be operated on. Such situations are just examples of when an estimation of the anticoagulant effect of the DOAC in question is called for.

The effects of DOACs on different coagulation assays are now well described (Baglin et al., 2012; Hillarp et al., 2011, 2014, 2018, 2020; Lindahl et al., 2011).

To summarize their findings, neither PT-INR nor aPTT (the activated Partial Thromboplastin Time) can, as routine analyses, be used to estimate DOAC intensity and even less to estimate DOAC-related bleeding risk in a specific patient.

The different DOACs influence PT-INT and aPTT differently. Rivaroxaban at peak concentrations can be expected to prolong aPTT, at lower concentrations to a lesser extent. The effect on PT-INR can vary, in general, PT Quick methods are more sensitive. Apixaban affects aPTT and PT-INR less than what is seen with rivaroxaban, whereas the effects of edoxaban are similar to those seen with rivaroxaban. Dabigatran affects aPTT already at low concentrations, PT-INR is less affected. For all DOACs there are substantial differences between different assays/reagents, making test results even more difficult to interpret. Also, all can, to a varying degree, cause interference with other coagulation-related assays, such as for lupus anticoagulant and antithrombin assays.

The problems are diverse. First, it is important for clinicians to understand they cannot judge the anticoagulant status of a DOAC patient by looking at PT-INR (and maybe aPTT) as they do with warfarin (or maybe heparin) treated patients. And second, there is a need for readily available tests (also outside university hospitals) that actually do give some guidance. These two problems also converge into a third: Interference from DOACs can lead to disguised erroneous results in coagulation-related testing. In Paper III and Paper IV, we address this.
4. CONTRIBUTIONS OF THIS THESIS—together towards the goal

“As you came from the breeze,
into dust you will go
What occurs in between,
you will strive hard to know!”

Ferdowsi
4.1 Introduction and Overview

The overall aim of this thesis was to investigate ways laboratory methods can contribute to improving oral anticoagulant treatment. Our common goal was to improve oral anticoagulant treatment, and joining the clinical and laboratory worlds and our forces (waving our wands!), we approached this from different perspectives:

1. To investigate into areas recently unveiled for further clarification

   **Paper I**, on genetic factors influencing warfarin treatment

2. To critically review the way we do things

   **Paper II**, on comparison between different warfarin treatment settings

3. To explore new ways to approach an unsolved problem

   **Paper III** and **Paper IV**, on a new way to measure DOACs in blood samples

In the following sections the papers are summarized and related to the common, overall goal.
4.2 Paper I: The VKORC1 gene and its influence on warfarin treatment

Objective
Investigate genetic factors as an explanation for differences in warfarin dose requirements and PT stability.

Material and Methods
Ninety-two warfarin-treated patients and 180 healthy controls participated in the study. The following methods were used:
- Haplotype analysis \((\text{VKORC1}^*1, \text{VKORC1}^*2, \text{VKORC1}^*3/3D, \text{VKORC1}^*4)\)
- DNA sequencing to detect mutations in the VKORC1 gene
- Analysis of warfarin enantiomers, PT and prothrombin
- Retrospective analysis of records (10 patients homozygous for \(\text{VKORC1}^*2\), 11 patients homozygous for \(\text{VKORC1}^*3\) or \(\text{VKORC1}^*4\))

Results
One new point mutation was detected (exon 2), unknown significance
Haplotype \(\text{VKORC1}^*1\) was not found in either of the groups.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>CONTROLS (%)</th>
<th></th>
<th>PATIENTS (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1n</td>
<td>2n</td>
<td>1n</td>
<td>2n</td>
</tr>
<tr>
<td></td>
<td>60.8</td>
<td>39.2</td>
<td>64.1</td>
<td>35.9</td>
</tr>
<tr>
<td>(\text{VKORC1}^*2)</td>
<td>63.1</td>
<td>36.9</td>
<td>60.9</td>
<td>39.1</td>
</tr>
<tr>
<td>(\text{VKORC1}^*3)</td>
<td>77.0</td>
<td>23.0</td>
<td>75.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 2. Haplotype frequencies (from Paper II).

Significantly lower levels of R-warfarin in patients with \(\text{VKORC1}^*2\).
Significantly lower warfarin dose in patients with \(\text{VKORC1}^*2\) v. \(\text{VKORC1}^*3\) or \(\text{VKORC1}^*4\).
More variation in PT in the \(\text{VKORC1}^*2\) group.
Conclusion
Homozygous VKORC1*2-patients show characteristics (lower dose, higher PT variability) that can indicate a higher risk with warfarin treatment and could be candidates for pretreatment genetic testing. Whether pretreatment testing is a feasible option depends on availability and cost.

Strengths and limitations
Multiple modalities were used (haplotype analysis, mutation analysis, analysis of warfarin in plasma, retrospective record analysis) with convergent results. Limited information was available on the participants regarding other factors of potential influence on the warfarin treatment (e.g., diet, physical activity, and other drug treatments).

Contribution to the overall goal
This study further clarifies genetic contribution to explain dose requirement variation in warfarin treatment. In particular, the genotype VKORC1*2/VKORC1*2 can be expected to show a phenotype with increased warfarin sensitivity, confirming that VKORC1 allele status adds information to what is known about CYP2C9 alleles and their impact on warfarin sensitivity (Takahashi et al., 2006; Yasar, 1999). The results are in line with later studies by others, e.g., in a large Swedish cohort described by Wadelius et al. (2009), where 12% of the variation in warfarin dose was estimated to be explained by the CYP2C9*2 and CYP2C9*3 alleles, 30% by a single nucleotide polymorphism in VKORC1, and 59% by a combined model using age, sex and drug interactions in addition to these two genes.

There is no reason to question that the information on a patient’s VKORC1 and CYP2C9 allele status is valuable in predicting the warfarin dose. The question is whether the benefit of having this information justifies the inevitable extra practical handling and cost that it entails. The jury is still out on this today (Fahmi et al., 2022). New in silico models, e.g., using Markov decision modeling and reinforcement learning as described by Anzabi Zadeh et al. (2023), can advance our knowledge. However, as they also conclude, even then the “clinician-in-the-loop approach” is a good thing (Anzabi Zadeh et al., 2023, p. 12).
4.3 Paper II: On organizing VKA treatment—
primary health care centers or centralized anticoagulation clinics?

Objective
To compare patient characteristics and INR results as a marker of therapeutic control in a cross-section material of warfarin-treated patients in ACCs and PHCCs in a first attempt to address the question of which is the optimal setting.

Material and Method
PT-INR results were collected in one week (February 2004) from 17 PHCCs and 3 ACCs in Östergötland, Eksjö, and Värnamo, all in Sweden. Information on the reason for warfarin treatment and the intended therapeutic range (TR) for each patient was collected from each caregiver (non-warfarin treated patients were excluded).

Results
Patient characteristics in Table 3 and Table 4.

<table>
<thead>
<tr>
<th>Location</th>
<th>PT-INR tests</th>
<th>Range stated</th>
<th>Cause stated</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHCC Östergötland</td>
<td>499</td>
<td>480</td>
<td>495</td>
</tr>
<tr>
<td>PHCC Eksjö</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td><strong>PHCC total</strong></td>
<td><strong>564 (289/275)</strong></td>
<td><strong>545 (282/263)</strong></td>
<td><strong>560 (289/271)</strong></td>
</tr>
<tr>
<td>(men/women)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC Östergötland</td>
<td>470</td>
<td>470</td>
<td>464</td>
</tr>
<tr>
<td>ACC Eksjö</td>
<td>58</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>ACC Värnamo</td>
<td>399</td>
<td>399</td>
<td>393</td>
</tr>
<tr>
<td><strong>ACC Total</strong></td>
<td><strong>927 (540/387)</strong></td>
<td><strong>927 (540/387)</strong></td>
<td><strong>915 (531/384)</strong></td>
</tr>
<tr>
<td>(men/women)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ACC = Anticoagulation clinics, PHCC = Primary health care centers.**

Table 3. Number of PT-INR tests (unique patients), known/reported TR, and reason for treatment (from Paper II).
In relation to their individual intended TR, 72% of the PHCC patients and 66% of the ACC patients were within TR (p<0.05, Chi² test).

More PT-INR tests were above the intended TR in the ACCs compared to the PHCCs (15% v. 11%, p<0.05, Chi² test) and overall in women more than men.

The PHCCs had significantly older patients and more men.

A higher proportion of male patients were within TR compared to women.

A higher proportion of male patients were within TR in the PHCC v. ACC setting.

No significant differences were found between settings for female patients.

**Conclusion**

Both settings show good therapeutic control. Limitations preclude definite conclusions about differences, but from this cross-section material, the PHCC setting appears to achieve at least as good therapeutic control as the ACC. A prospective and randomized head-to-head study would be needed for improved comparison.
**Strengths and limitations**
The relatively large number of patients is a strength, as is the real-life approach. The obvious limitations are the lack of more comprehensive data on patient characteristics and the fact that we cannot wholly exclude referral bias. As for the general applicability of the results, there is an obvious limitation: at the time of the study, both settings had a long experience of warfarin treatment management. As I see it, this study would not be feasible to replicate today, as by now there are few PHCCs with both the experience and ongoing treatments.

**Contribution to the overall goal**
The organization of warfarin treatment surveillance can impact the quality of the treatment. This study, even considering its limitations, gave rise to interesting questions about the underlying strength in each setting (e.g., a larger patient cohort in an ACC but personal patient knowledge beyond formal records in the PHCC). A follow-up, randomized study was planned and well underway when this cross-section study was concluded. Unfortunately, the randomized study was never completed. The reason for this was, at least in part, decisions made in our region to centralize warfarin treatment to ACCs, leading to a swift transition to the centralized model. Maybe this decision was correct, or even necessary, from an organizational perspective, and we have no doubt whatsoever that warfarin patients are very well taken care of at the ACCs. But still, when someone says it had to be done because the quality of treatment was inferior in the PHCCs, we have our doubts.
4.4 Paper III: A new prothrombin time method as a tool for measuring DOACs

**Objective**
To explore a PT-INR-method modification in the light of an identified need of a point-of-care (POC) method for measuring DOACs in patient samples.

**Material and Methods**
A commercially available PT-INR POC instrument was used, with modifications to sample dilutions (1:11 and 1:41 in addition to standard 1:21) as different dilutions were expected to show different interference from DOAC content in the sample. A PT ratio was calculated as a quotient between the INRs for the 1:11 and 1:41 dilutions. Concentrations of dabigatran, rivaroxaban, and apixaban were analyzed at the central laboratory of the University Hospital in Linköping for reference. Spiked plasma samples and authentic patient plasma samples were tested. In addition whole blood samples from three patients on rivaroxaban treatment were analyzed as a feasibility test.

**Results**
PT ratio in relation to central laboratory drug concentration measurements in patient samples in Figure 7, a) dabigatran (r = 0.89), b) apixaban (r = 0.91), c) rivaroxaban (r = 0.94).
Detection limit was 58 μg/L for dabigatran, 19 μg/L for rivaroxaban and 81 μg/L for apixaban.
Feasibility test with whole blood samples showed a marked reduced sensitivity (35%) compared to plasma analysis.

**Conclusion**
This modified PT-INR POC method based on a dilution-based PT ratio shows promise to be able to measure DOACs in patient plasma samples.
Figure 7 a-c). DOAC concentrations v. PT ratio.
**Strengths and limitations**

The correlations between PT ratio and reference drug concentrations are robust. Simple modifications were needed to an existing POC method, being easy to replicate. Limited sensitivity to lower concentrations, especially for dabigatran, can limit use in some clinical situations.

**Contribution to the overall goal**

This newly developed method can contribute to solving the problem of the lack of a quick and readily available POC method to detect the presence of DOACs and measure their concentrations in different clinical situations.
4.5 Paper IV: Avoiding erroneous laboratory results caused by DOAC interferences with coagulation analyses

Objective
To evaluate a now commercially available test based on the PT ratio method previously described in Paper III, on its ability to detect the presence of FXa inhibiting DOACs in plasma samples, at levels causing interference and possibly false results.

Material and Methods
MRX PT DOAC assay, designed to run on central laboratory equipment (not POC). FXa assays, assays for lupus anticoagulants (LA), and antithrombin, all according to routine analyses at the central laboratory at Linköping University Hospital. Plasma samples from healthy volunteers for reference interval, biobanked plasma samples for FXa inhibitor detection level determination, plasma samples from patient thrombophilia investigations for detection testing. LA positive tests were re-tested after treatment with DOAC-Stop™ to eliminate potential DOAC interference, and interference was concluded when there was a significant change in results.

For reasons of safety, the crucial requirement for this screening tool is that a negative result (i.e., no interference detected) is reliable, i.e., there are no false negatives. We want to process negative samples according to routine without risking interference that causes erroneous results. False positives, i.e., samples incorrectly marked as potentially interfering, would lead to extra testing but no errors. Based on this requirement, we focused on the negative predictive value for the tests we evaluated (Trevethan, 2017).

Results
The MRX PT DOAC detected all samples with levels of apixaban or rivaroxaban known to cause interference. The results from different subgroups of 315 patient thrombophilia investigation samples were evaluated.
The negative predictive value was 0.90 or above (95% confidence interval with a minimum of 0.70) for excluding analysis interference with LA and antithrombin analyses and for excluding DOAC treatment according to medical records. LA interference was indicated in only a total of 9 DOAC samples; all were detected by the MRX PT DOAC. For antithrombin, 11 DOAC samples indicated interference, and 6 were detected by the MRX PT DOAC.

**Conclusion**
The MRX PT DOAC has shown an ability to detect potentially interfering DOAC levels in plasma samples and is thereby a potential screening tool for excluding such analytical interference. The study included a relatively large number of tests. However, relatively few samples indicated interference, leading to small subgroups. Confirmation of the results in a larger number of interfering samples would be beneficial for the validity of the results. The divergent results for the detection of analytical interference with the antithrombin analysis need further attention to seek possible sensitivity improvements.

**Strengths and limitations**
Several aspects of the MRX PT DOAC ability were evaluated, which is a strength, as is a large material in total. Limitations are the limited number of patients in the smaller subgroups, as well as not having actual drug concentrations for reference when evaluating the detection of ongoing treatment.

**Contribution to the overall goal**
The MRX PT DOAC has the potential to contribute to safety for patients treated with DOACs by detecting potentially interfering DOAC levels in plasma samples, a problem that can otherwise easily be overlooked. Additional testing to confirm the results will be a challenge and will probably call for a new strategy to increase the ratio of interfering tests in such study material.
4.6 Ethical considerations

All studies were approved by the Swedish Ethics Review Authority/Regional Ethics Committee. Below is a summary of ethical considerations.

**Paper I**
The treated patients can have been subjected to an extra venous blood sampling (sometimes routine sampling can be capillary) and were not provided with a report of their genetic results. The control DNA samples consisted of anonymous biobank material previously collected for research purposes, including genetic studies.

The venous sampling (if extra) does not pose a significant discomfort to the patients (all adults) in the study, and the risk of complications is considered low. All patients were already on stable anticoagulant treatment; thus, information on individual genetic profiles was not considered to contribute to their care.

**Paper II**
No extra blood sampling was done. Possible harm to be caused by this study could be patient concerns about the results for a specific treatment setting. We considered this to be a small risk.

**Paper III**
Previously collected, biobanked patient plasma samples were used for drug concentration determinations. No extra blood sampling was done for the collection of blood donor plasma.

The venous sampling does not pose a significant discomfort to the patients (all adults) in the study, and the risk of complications is considered low. We concluded that without clinical grounds for determining the drug concentrations, there is no definite advantage for the patient (or the treating physician) to be provided with that information. On the contrary, levels may lead to unjustified treatment modifications. Also, the samples were biobanked and did not reflect current patient status.
A CLINICIAN, LABORATORY ANALYSES, AND ANTICOAGULANT TREATMENT

Paper IV

The treated patients in the reference material can have been subjected to an extra venous blood sampling (sometimes routine sampling can be capillary). Previously collected, biobanked patient plasma samples were used for detection-level analysis. No extra venous sampling was done for the collection of thrombophilia test samples. No study results were reported back to the patient or the treating physician.

The venous sampling (when extra) does not pose a significant discomfort to the patients (all adults) in the study, and the risk of complications is considered low. As for the Paper III study, we concluded that without clinical grounds for determining the drug concentrations, there is no definite net advantage for the patient (or the treating physician) to be provided with information on drug concentrations. Also, the biobanked samples did not reflect current patient status. As for the interference results, there is no benefit for the patient or the treating physician to be provided with them.

Conclusion

We found no significant risks for the study participants in this thesis. There was no immediate advantage for the participants, but the conclusions from the studies may contribute to improved anticoagulant treatment in the future.
“Don’t adventures ever have an end?
I suppose not.
Someone else always has to
carry on the story.”

Bilbo Baggins
In The Fellowship of the Ring
by J.R.R. Tolkien
This thesis spans almost two decades. Naturally, the findings must be related to their time. As all scientific results always must be. Some of the references referred to in this thesis were written a century ago, some at the time of World War II, some were published just weeks ago when this thesis goes to print (and a few have their publication date in early 2024!). If we can relate them to their time, there are lessons to be learned from all of them.

This thesis will not change the world, but it was indeed written. Science is the book of books of never-ending knowledge-seeking notes handed on from generation to generation. Each of us contributes to it, some more than others. But be assured, it is only when we do share what we know, not discarding the maybe less important findings, that we can join all forces and be true magicians.
Acknowledgements

“All’s well, that ends better.”

Hamfast Gamgee (Gaffer)
In The Lord of the Rings
by J.R.R. Tolkien
This is the part where I really, really want to thank you all who have contributed to this book, directly or indirectly. But as you can imagine, over all these years there have been many of you - and I want to remember you all here, but please forgive me in advance if I don’t. A warm thank you from me to you all!

With all that said, first and foremost on my list is my main supervisor Tomas Lindahl. My greatest thanks are really yours. Without your never-ending encouragement, patience, and understanding for all the things that sent me on detours, this book would still not have been written. I have learned so much from you over the years, both about research (and how to stay true to your research ethics, no matter what!) and how to solve clinical mysteries with the help of laboratory methods. You are indeed a true magician! I think of you as my friend, and I hope for many more conversations ahead.

Thank you, my current co-supervisors Srinivas Uppgunduri and Margareta Holmström - I am so happy you believed in me! You have both contributed in so many ways to help me keep up the hope of finalizing this. Your clinical knowledge is also beyond invaluable, Margareta! I also want to thank Sven Karlander, my previous co-supervisor, for once-upon-a-time helping me find a starting point for this journey. Nina Nelson Follin, together we saw some good and bad sides of research all those years ago, but thank you for all you taught me about what really matters, in both research and life. Delayed delivery, and on a slightly different subject than the one we worked on, but finally a book!

So many researchers and PhD students have contributed to making this long journey worth it in so many different ways. Thank you to my co-author Abdimajid Osman, for being a perfect chemist-match for clinician-me, and also for all the interesting discussions—and nice figures, thank you for providing them! And Kenny Hansson, thank you so much for finding my favorite coagulation system on a file, and giving it to me! Thank you also, Camilla Enström, Peter Söderkvist, and Mats Rånby for
co-authoring and contributing with invaluable laboratory-technical knowledge beyond mine.

Tomas Lindahl’s research group over the years, thank you all so much, all of you, for including me as a clinician in your lab world! Sofia Ramström for your brilliant thinking and way of explaining so that even a clinician can understand, I miss you here in Linköping! Nahreen Tynngård for your kindness and research endurance, Lars Faxälv for being such an uncomplicated roommate back then, Martina Nylander and Karin Öberg for being nice to me! A special thank you to three more physicians in the research group: Roza Chaireti, for both research cooperation and co-authoring articles beyond the scope of this thesis, and all our clinical hours together, I miss you too here! Niklas Boknäs for your sharp mind and kind spirit, and for being a bit late and disorganized, making it easier for me to be not-so-perfect either. Mikael Lund, my most recent co-author for your input. Last, but not in any way least!, Kerstin Gustafsson for all your skilled lab work on research samples through the years, and Ewa Lönn and Karin Erlin for mastering all those big machines, and patiently explaining to me the tricks of the lab trade.

Maurice Devenney, thank you for your brilliant skills in improving our English writing, including the mysterious hyphen, en dash, and em dash—I’m getting there, did you see?, but also for those kind words when I needed them the most.

In the clinical and more administrative, less research part of my work life I have to thank current and previous bosses for making this possible after all, especially Henrich Wilander, and now Niclas Hilding. And for making everything administrative so much easier, thank you Helene Hall! Around me at my clinical home, the Department of Acute Internal Medicine and Geriatrics, and also at my other clinical home at Karolinska University Hospital, I have so many colleagues (magicians!) of all sorts that I am grateful for having by my side, or having had, for some that had to leave (I miss you!). Thank you especially for all the stimulating discussions, and for never forgetting what is really important in relation to patients and co-workers. And here comes: A very sincere thank you, from deep in my heart, to all of you who would never refer to a patient as “it”, and also understand why.
Entering this last page, I want to send some very special thanks to some of you who are important to me outside of the medicine area. All you wonderful and wise animal lovers at the Animal Psychology and Biology Bachelor Program, thank you for having me! Mimmi, Elna, and Karin—thank you for all those inspiring hours of statistics and so much more! And also Felicia, Nasra, and Paulina—the statistician-magicians of tomorrow, you were so kind to me when I had to write a book instead of finishing up the lab report!

There are some friends out there who have had to wait too long for me to call, but you don’t give up, thank you so much—Ann-Christine, Angelica, and others, I will be back! I also want to send the same message to Kerstin Backman and all my other Quaker Friends: I will be back! You live in my heart and soul, just close to the light.

Finally, my family, to whom this book is dedicated. My mother and father had to leave, the way life, and its ending, happens to us. You defined me in so many good ways, always believing in me, I am forever so grateful for that. My brother Jan Arbring, thank you for holding on to that sibling bond, also now when we are the eldest.

My closest family, my wonderful children Joel Arbring, Theresia Arbring Sjöström, and Anton Arbring, thank you for being exactly the way you are. And Theresia, thank you for the beautiful flowers on the cover of this book. Your book was far more exciting than this, and I am so proud to see the researcher you have become. Fredrik Arbring Sjöström, I am so happy to have you, and your parents Pirjo and Staffan Sjöström, in our family. You and Theresia have two wonderful children, our granddaughters, they mean the world to me! To you too, Idun and Lo - I really hope I will be seeing more of you again now this book has been written!

Reibert - my love and partner for so many years we can hardly count them - thank you for putting up with me, for believing in me, and for all the dog walks! And Pixel and Leia, endless love on four legs each, I hope you know how much we love you both, and how hard we try to be your best humans. You don’t have to think about reinforcement learning, you are the best teachers. In that, too.
This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”

Winston Churchill
References


64
PMID - 18955670


https://doi.org/10.1016/j.tmrv.2019.08.004

https://doi.org/10.1016/j.jtha.2023.06.036

https://doi.org/10.1007/s11096-022-01386-8

https://doi.org/10.1016/j.cca.2009.10.016

https://doi.org/10.1016/j.jtha.2023.04.021

https://doi.org/10.1160/th05-04-0290 PMID - 16270629

Giugliano, R. P., Ruff, C. T., Braunwald, E., Murphy, S. A., Wiviott, S. D., Halperin, J.


Osman, A. (2007). *Studies on warfarin treatment with emphasis on inter-individual variations and drug monitoring.*


https://doi.org/10.1056/nejmoa1009638 PMID - 21830957


https://www.R-project.org/


https://doi.org/10.1056/nejmoa044503 PMID - 15930419


https://doi.org/10.1056/EVIDoa2300067

https://doi.org/10.1001/archinte.167.3.239 PMID - 17296878


https://doi.org/10.1016/j.thromres.2009.03.007 PMID - 19423152

https://doi.org/10.1023/A:1012742628628


Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

https://doi.org/10.3384/9789180753470
Two Worlds, One Goal: A Clinician’s Perspective on Laboratory Analyses in Anticoagulant Treatment

Kerstin Arbring