Determinants of adverse events during oral anticoagulant treatment

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To Maria, Adrian and Frank
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ABSTRACT

Treatment with oral anticoagulation is highly effective in reducing the burden of thromboembolic complications in several clinical conditions. The number of patients receiving oral anticoagulation is growing steadily. In Sweden about 1.5 percent of the population receives treatment. Although the treatment is highly effective in preventing thromboembolic complications, it is also associated with a substantial increase in the risk of bleeding. In clinical practice every physician has to balance the potential benefit of treatment against the risk of bleeding complications in the individual patient.

To aid in this decision making, risk scores addressing the likelihood of thromboembolic events, as well as the risk of bleeding complications, have been developed. These scores are imperfect and, to some degree limited by the fact that the risk factors predictive of thromboembolic events are also often associated with bleeding complications. The addition of biomarkers has the potential to increase the predictive ability of risk scores and further enhance the net benefit of oral anticoagulant treatment in the individual patient. In this thesis several potential biomarkers for thromboembolic and haemorrhagic complications of anticoagulant therapy have been investigated in a longitudinal cohort study of 719 patients with a median follow-up time of 4.2 years.

Thrombomodulin is a key component in the generation of activated protein C and hence, a coagulation inhibitor. Conversely, it is also a key component in the inhibition of fibrinolysis by activation of trombin-activated fibrinolysis inhibitor. In warfarin-treated patients we demonstrate that thrombomodulin predicts an increased risk of bleeding complications, but not cardiovascular events. Thus, thrombomodulin has potential as a biomarker specifically for bleeding complications.

Von Willebrand factor plays a central and intricate role in the aggregation of platelets and low levels of VWF have been associated with bleeding as a manifestation of von Willebrand’s disease. In our study we noted that high levels of von Willebrand factor predict an increased risk of cardiovascular as well as all-cause mortality, possibly as an expression of endothelial dysfunction. We also noted that high levels of VWF seem to be associated with serious bleeding complications.

Decreased renal function is usually measured by an increase in the levels of creatinine and cystatin C, or a decrease in the calculated glomerular filtration
rate. A decrease in kidney function is regarded as a marker of an increased risk of bleeding complications. We investigated all the mentioned markers of kidney function and no association with bleeding complications became apparent. However, a clear association between a decrease in kidney function and mortality was noted. Our findings indicate that the emphasis on impaired kidney function as a risk marker needs to be shifted from bleeding complications toward thromboembolic events.

Fibrinolysis is important in containing coagulation and several constituents of the fibrinolytic pathway have been shown to predict cardiovascular events and mortality. We found that fibrinolytic factors seem to predict cardiovascular events in patients with oral anticoagulation and that D-dimer also predicts bleeding complications.

In conclusion, we have found several biomarkers which exhibit different predictive abilities in patients with oral anticoagulation. It is likely that biomarkers, either alone, in combination, or as ancillary components of risk scores, can contribute to improved risk stratification in patients with oral anticoagulation.
Behandlingen med blodförtunnande mediciner ökar och idag behandlas 1.5% av befolkningen. Den dominerande substansen är warfarin men nya läkemedel har utvecklats och introduceras nu i klinisk medicin. Nytta av blodförtunnande medicinering för att förebygga blodproppar är otvetydig vid ett flertal tillstånd. All blodförtunnande medicinering, både etablerade och nya, är förknippad med en ökad risk för allvarliga blödningskomplikationer.

Idag används ett flertal riskmarkörer för blödning respektive blodproppar under behandling med antikoagulantia för att värdera nytta med behandling för patienten. Värderingen av riskerna för den enskilde patienten är ändå svår, då merparten av riskfaktorerna är associerade både till en ökad risk för blödning, men även en ökad risk för blodproppar. Syftet med den här avhandlingen var att hitta nya biomarkörer med potential att förbättra riskvärderingen vid behandling med blodförtunnande medicin.

Avhandlingen bygger på en population bestående av 719 patienter med blodförtunnande behandling vilka följes under drygt 4 år. Samtliga patienter lämnade blodprover innan registreringen av blödningskomplikationer, blodproppar och dödsfall inleddes. Associationen mellan blödningar, död och kardiovaskulära händelser och nivåer av thrombomodulin, von Willebrand faktor, cystatin C, kreatinin, D-dimer, tPA och tPA/PAI-1 komplexet undersöktes.

Thrombomodulin är en central komponent i hemostasen. Höga nivåer av thrombomodulin var i vår population av waranbehandlade patienter kopplade till en ökad risk för blödningskomplikationer men inte för kardiovaskulära händelser.

von Willebrand faktor spelar en viktig roll i processen när kroppens blodplättar aggregerar. I detta material var höga nivåer av von Willebrand faktor associerade inte bara till kardiovaskulära händelser och död utan även till blödningskomplikationer hos waranbehandlade patienter.

Nedsatt njurfunktion, uttryckt som höga nivåer av cystatin C eller kreatinin eller lågt eGFR, uppvisade en koppling till framtida risk för kardiovaskulära händelser och död. Något samband med blödningskomplikationer kunde inte påvisas.
D-dimer som en markör för fibrinolysen, kroppens blodproppsupplösande system, visade en association både till blödningar men även till kardiovaskulära händelser.

# ABBREVIATIONS

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<td>AE</td>
<td>arterial emboli</td>
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<tr>
<td>APC</td>
<td>activated protein C</td>
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<td>APTT</td>
<td>activated prothrombin time</td>
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<td>AT</td>
<td>antithrombin</td>
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<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>CYP</td>
<td>cytochrome P</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
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<tr>
<td>ECG</td>
<td>electrocardiogram</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPCR</td>
<td>endothelial protein C receptor</td>
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<tr>
<td>FDP</td>
<td>fibrin degradation products</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>HR</td>
<td>hazard ratio</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>INR</td>
<td>international normalized ratio</td>
</tr>
<tr>
<td>ISI</td>
<td>international sensitivity index</td>
</tr>
<tr>
<td>LH-</td>
<td>likelihood ratio negative</td>
</tr>
<tr>
<td>LH+</td>
<td>likelihood ratio positive</td>
</tr>
<tr>
<td>LMWH</td>
<td>low molecular weight heparin</td>
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<tr>
<td>NPV</td>
<td>negative predictive value</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OAC</td>
<td>oral anticoagulant</td>
</tr>
<tr>
<td>PAI 1</td>
<td>plasminogen activator inhibitor type 1</td>
</tr>
<tr>
<td>PC</td>
<td>protein C</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PTT</td>
<td>prothrombin time</td>
</tr>
<tr>
<td>sTM</td>
<td>soluble thrombomodulin</td>
</tr>
<tr>
<td>TAFI</td>
<td>thrombin activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TAT</td>
<td>thrombin-antithrombin complex</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TIA</td>
<td>transient ischemic attack</td>
</tr>
<tr>
<td>TM</td>
<td>thrombomodulin</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue type plasmin activator</td>
</tr>
<tr>
<td>tPA/PAI-1 complex</td>
<td>the complex formed between tPA and PAI-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>TXA₂</td>
<td>tromboxane A₂</td>
</tr>
<tr>
<td>uKA</td>
<td>urokinase type activator</td>
</tr>
<tr>
<td>VKA</td>
<td>vitamin K antagonist</td>
</tr>
<tr>
<td>VKOR</td>
<td>vitamin K epoxide reductase</td>
</tr>
<tr>
<td>VTE</td>
<td>venous thromboembolism</td>
</tr>
<tr>
<td>WP</td>
<td>Weibel-Palade bodies</td>
</tr>
<tr>
<td>WRN</td>
<td>warfarin-related nephropathy</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
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The thesis is based on the following papers:


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INTRODUCTION

Treatment with oral anticoagulants

In the early 1920’s, Schoefield investigated an outbreak of a bleeding disorder among cattle in North America. He found that the disease occurred in animals grazing on yellow sweet clover (Melilotus officinalis) containing dicumarol, a by-product of coumarin resulting from mold infestation of the plant.\(^1\) Dicumarol possesses a potent anticoagulant effect and several analogues were later synthesized. One of the analogues was named warfarin,\(^2\) the principle anticoagulant drug in healthcare for more than 5 decades, whose use has increased steadily. Despite its advantages, no other drug is responsible for as many adverse events. Significant resources are allocated to monitor and adjust treatment with warfarin. Additionally, anticoagulant therapy is initiated in patients with increasing age and multiple co-morbid conditions leading to a higher risk of bleeding complications. In Skellefteå, a community with a population of approximately 75,000, over 1,400 patients are presently receiving oral anticoagulants (OAC).

Vitamin K antagonists (VKA), predominantly warfarin, are the preferred oral anticoagulant for the prevention of stroke in patients with atrial fibrillation.\(^3\) However, this is likely to change since several new pharmacological therapies are being introduced.\(^4\) \(^5\) \(^6\) The new anticoagulant drugs will not change the clinical dilemma of balancing the desire to decrease the risk of thromboembolic events against the increased risk of bleeding complications.

Haemostasis

Hemostasis represents a balance between procoagulant and anticoagulant factors which has evolved over time to protect against fatal haemorrhage and thrombosis. The main components of haemostasis are platelets, coagulation factors, fibrinolysis and vessel integrity.\(^7\) Coagulation is initiated when a vessel is injured and cells bearing tissue factor (TF) are exposed to plasma. Platelets adhere to von Willebrand factor (VWF) at the site of injury \(^8\) \(^9\) and aggregate due to released ADP and Tromboxane A\(_2\) (TXA\(_2\))\(^10\) resulting in a primary haemostatic plug. Fibrinogen is then transformed into fibrin by the coagulation cascade located on activated cell surfaces.\(^11\) A local fibrinolytic response with the activation of plasmin helps to restrict coagulation at the site of injury,\(^12\) while activation of the protein C anticoagulant pathway turns off further thrombin generation in a thrombomodulin dependent process. The end result is the formation of a durable sealing of a damaged vessel without the formation of excess thrombus, reflecting a haemostatic process.
in balance. Definitive haemostasis is then achieved by the addition of fibrin produced by the coagulation cascade. In order to contain coagulation, a local fibrinolytic response with the formation of plasmin and degradation of fibrin is important.

**Coagulation**

Coagulation is a process often represented by an organized succession of reactions resulting in the formation of thrombin from prothrombin and the subsequent formation of fibrin from soluble fibrinogen. The process of coagulation is often described by a waterfall cascade with stepwise activation of factors of the coagulation. The cascade model supports the existence of an intrinsic and an extrinsic pathway leading up to a common pathway described in Figure 1.\(^2\)

![The cascade model of coagulation](image)

**Figure 1.** The cascade model of coagulation

Assessment of the different pathways can be made by the activated prothrombin time (APTT), which reflects the function of the intrinsic
pathway, and the prothrombin time test (PTT), which reflects the extrinsic pathway.\textsuperscript{13}

Over time it has become evident that there are clinical observations that are hard to explain by applying the cascade model of coagulation in vivo. For instance, it is not clear why factor VII deficiency should lead to bleeding when the intrinsic pathway is unaffected.\textsuperscript{13} An alternative, cell-based model of coagulation has therefore been suggested and is described in Figure 2.\textsuperscript{14}

\textbf{Figure 2.} Overview of the cell-based model of haemostasis

This model regards haemostasis as occurring in three phases, initiation, amplification, and propagation.\textsuperscript{14} The initiation phase is an ongoing activation of small amounts of coagulation factors VIII, X and II by TF localized in extravascular cells. Since the activated coagulation factors are separated from the other necessary components of hemostasis by an intact vessel, no clots are formed. When a vessel is damaged, larger components of the haemostasis such as VWF, platelets, and factor VIII are able to reach the small amounts of thrombin generated in the initiation phase. The thrombin activates platelets and factor V and VIII and this phase is called the amplification phase. In the third and final stage, entitled the propagation phase, factors IXa and VIIIa combine on the surface of activated platelets
and activate factor X, which then combines with factor V and activates large amounts of thrombin. Thrombin then acts on fibrinogen producing fibrin monomers which then polymerize and form a stable fibrin clot. Surplus thrombin that escapes the site of injury is neutralized in the circulation by antithrombin, forming enzymatically inactive thrombin-antithrombin complexes (TAT) which are rapidly cleared from the circulation, mainly in the liver. Alternatively, thrombin can become bound to endothelial surface Thrombomodulin (TM).

When thrombin is bound to thrombomodulin its substrate specificity changes from fibrinogen to protein C. The thrombin-thrombomodulin complex then catalyzes the conversion of protein C to activated protein C (APC) which inactivates factors Va and VIIIa and thereby down regulates the coagulation cascade.\textsuperscript{15}

### Fibrinolysis

In order to dissolve clots formed in vessels and balance the deposition of fibrin with its subsequent removal, the fibrinolytic system is essential. This allows for concentration of coagulation activity at the site of injury while maintaining fluidity elsewhere in the vascular system.\textsuperscript{11, 12, 16} Initiation of fibrinolysis begins with the activation of plasminogen to plasmin by the plasmin activators in the presence of fibrin. Plasmin activators bound to fibrin are protected from inactivation by alfa-2-antiplasmin and Plasminogen Activator Inhibitor type 1 (PAI-1), but circulating plasmin activators are rapidly inactivated.\textsuperscript{11} There is a link between coagulation and fibrinolysis in the form of thrombin activated fibrinolysis inhibitor (TAFI).\textsuperscript{17, 18} Thrombin bound to thrombomodulin inhibits fibrinolysis by activating TAFI to TAFIa, which modifies fibrin thereby reducing its co-factor activity in the activation of plasminogen.\textsuperscript{15} Thrombomodulin also changes the substrate specificity for thrombin so that instead of acting on fibrinogen, thrombin in complex with thrombomodulin converts protein C into APC. In conclusion, an increased activity of coagulation leads to a suppression of fibrinolysis.\textsuperscript{15}
### Figure 3. Schematic view of fibrinolysis

#### Haemostasis and inflammation

There is a bidirectional relationship between inflammation and coagulation.\(^\text{19}\) Inflammation affects coagulation mainly by increasing the levels of cytokines like interleukins 6 (IL-6)\(^\text{20}\) and tumour necrosis factor-alpha (TNF-\(\alpha\)).\(^\text{21}\) The expression of TF on endothelial cells seems to be IL-6 dependent\(^\text{20}\) and TF has also been found on inflammatory cells in arteriosclerotic plaques.\(^\text{22}\)

Several inhibitors of coagulation are affected by inflammation. Antithrombin (AT) concentrations drop during an inflammatory state due to increased thrombin generation and, thereby, increased consumption. AT levels also fall as part of the acute phase response\(^\text{23}\) with decreased synthesis in the liver.\(^\text{24}\) Levels of protein S\(^\text{25}\) and protein C drop during inflammation, mainly due to consumption, lower synthesis, and increased degradation.\(^\text{24, 26}\) Similarly, downregulation of thrombomodulin in conjunction with increasing levels of cytokines leads to decreased levels of APC.\(^\text{27, 28}\) The changes in the levels of AT and activated protein C leads to a diminished inhibition of coagulation.

During inflammation, secretion of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) increases, as well as the generation of PAI-1. The latter is a more sustained response and the net
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effect on fibrinolysis is inhibition. In conclusion, inflammation leads to an increased activation of coagulation mediated mainly by an increased expression of TF. There is also a decrease in the inhibition of coagulation as well as decreased fibrinolysis during inflammation. These changes results in an imbalance of haemostasis leading to a prothrombotic state.

Several components of haemostasis affect the inflammatory response. Factor Xa, thrombin, and fibrin activate endothelial and mononuclear cells and, secondarily, the synthesis of IL-6 and IL-8 increases. Antithrombin acts as an inhibitor of inflammation in several different ways. The main pathway is by inhibiting thrombin. AT also induces prostaglandin release from endothelial cells which inhibits platelet activation and decreases endothelial synthesis of cytokines. APC has a well established role as a modulator of inflammation in sepsis. The effects of APC on inflammation is thought to be mediated via binding to epidermal growth factor receptor (EGFR), leading to transcriptional effects that are anti-inflammatory. In Figure 4 the effects of haemostasis and inflammation on each other is shown.

Figure 4. Bidirectional interactions between inflammation and haemostasis.
Warfarin therapy

Warfarin is a coumarin derivative which is administered orally as a racemic mixture of two isomers and absorbed from the gastrointestinal tract with almost 100% bioavailability. Maximum blood concentrations are reached after 90 minutes of oral administration and the half-life is between 36 and 42 hours. Warfarin inhibits the production of vitamin K-dependent coagulation factors by interfering with the cyclic interconversion of vitamin K and its 2,3 epoxide. Vitamin K is a cofactor for the posttranslational carboxylation of glutamate residues to γ-carboxyglutamates on the N-terminus of several vitamin K-dependent coagulation factors (II, VII, IX and X). The coagulation factors require γ-carboxylation for their biological activity and carboxylation, in turn, requires the reduced form of vitamin K (vitamin KH₂) which is linked to the oxidation of vitamin KH₂ to vitamin K epoxide. Vitamin K epoxide is then recycled through two reductase steps. The first step is sensitive to vitamin K antagonism, while the other step is not. Supplementation with vitamin-K renders substrate available for the insensitive enzyme and the effect of the VKA is thereby reversed. Supplementation with large doses can cause VKA resistance for several days due to accumulation of vitamin K in the liver.

The anticoagulant factors protein C and S are also diminished by warfarin therapy and, due to their shorter half life in plasma than vitamin K-dependent coagulation factors, a procoagulant state arises during the initiation of warfarin therapy. This needs to be considered during the initial phase of warfarin therapy, not only during treatment of venous thromboembolism, but even during treatment of atrial fibrillation.

Monitoring of treatment

The dose response of warfarin is influenced by genetic as well as environmental factors. Polymorphisms of the gene encoding for specific cytochrome P450 enzymes (2C9) and mutations in the coding of the vitamin K epoxide reductase complex (VKORC1) affect the dose required for anticoagulant effect. Other drugs, dietary factors, as well as concomitant diseases can affect the warfarin response in an individual patient. In order to achieve an optimal warfarin treatment, monitoring is therefore needed.

Initially a prothrombin test (PT) expressed in seconds or as a ratio compared to normal subjects was used to monitor the effect of warfarin. The prothrombin test is prolonged by reduction in factors II, VII, and X and is performed by adding calcium and a phospholipid rich extract of tissue (thromboplastin) to citrated plasma. Preparations of thromboplastin vary.
in their responsiveness to warfarin \(^5\) and in order to adjust for this the international normalized ratio (INR) has been introduced. The responsiveness of a PT test could be measured as ISI values with an ISI value of 1.0 reflecting a highly sensitive test. Higher values represent a less sensitive test. From the ISI value an INR can be calculated using a standard preparation of thromboplastin and calibrating the local test to it.\(^5\) An INR can then be determined by the formula:

\[
\text{Log INR} = \text{ISI} \times \log_{\text{observed PT ratio}}
\]

Today most anticoagulation centres use the INR standard when monitoring warfarin treatment.

The optimal testing interval for monitoring the anticoagulant effect is not known, but it is likely that a more lengthy period between controls is adequate as demonstrated by Schulman et al.\(^5\)

**Optimal level of INR**

Several studies have investigated the optimal interval of INR for different indications. A level of INR between 2.0 and 3.0 is regarded as an optimal range for most indications.\(^5\) It has also been shown that an INR of 2.0 or greater not only reduces the frequency of stroke in patients with atrial fibrillation, but also the severity.\(^5\)

The exception to the above recommended INR interval is patients with prosthetic heart valves in which a value as high as 3.5 is recommended.\(^6\)

**Indications for treatment**

Warfarin is used in several conditions, but the most common indications are atrial fibrillation, deep venous thrombosis, and prosthetic heart valves.\(^4\)

**Atrial fibrillation**

Atrial fibrillation is a common disorder with an increasing incidence as age increases; the prevalence of atrial fibrillation has been shown to be about 0.95 - 3.0%.\(^6\) Atrial fibrillation significantly increases the risk of stroke and treatment with oral anticoagulants is highly effective in preventing
stroke in this group of patients. The relative risk reduction has been reported as being as high as 71%.

**Treatment and prevention of venous embolism**

Anticoagulation has been shown to be highly effective in the treatment of pulmonary embolism as well as in the treatment of deep venous thrombosis in the extremities. Warfarin is effective in preventing a recurrent thrombosis both in patients with pulmonary embolism and venous thrombosis. The optimal duration of treatment is not known and is likely to differ between a first episode of thrombosis versus a recurrent episode of thrombosis, and, likewise, between a provoked versus an unprovoked thrombosis. Other factors that could affect the duration of treatment are malignancy, thrombophilia and the extent of thrombosis.

Warfarin has also been studied as a preventive treatment after orthopaedic and gynaecologic surgery.

**Prosthetic heart valves**

The risk of thromboembolic complication in patients with mechanical heart valves is substantial, being as high as 12% per year in the aortic position and up to 22% per year for mitral valves. These statistics are likely to be lower in patients with more modern mechanical valves. Warfarin reduces the risk of thromboembolism in patients with prosthetic heart valves and represents standard therapy in all patients with mechanical heart valves today. The optimal target INR in patients with prosthetic heart valves has been investigated in several studies. Even an extreme target value of INR (9.0) does not offer additional protection against thromboembolism over a moderate target INR (2.65). It is possible that the addition of low dose of aspirin could enhance to the preventive effect of warfarin treatment, but at the expense of more bleeding complications.

In pregnancy, warfarin is associated with an increased risk of embryopathy and is sometimes substituted with heparin or low molecular weight heparins (LMWH). In patients with prosthetic heart valves the latter approach have been associated with a dramatic increase in valvular thrombosis.

**Other indications**

Warfarin is also established as a therapeutic option in several other conditions. In idiopathic pulmonary hypertension it is thought that warfarin prohibits the formation of small thrombi in the pulmonary arterial tree,
thereby limiting the progression of the disease. Clinical studies supporting this notion are sparse. Based on a systematic analysis in 2006 it is clear that large prospective studies are lacking and that the beneficial effect of warfarin in idiopathic pulmonary hypertension needs to be viewed with some caution.

In patients with acute coronary syndromes warfarin have been proven beneficial, both as a single therapy and in combination with aspirin. The effect on risk reduction with the addition of warfarin to aspirin is substantial even when compared with the addition of clopidogrel to aspirin. Warfarin after a myocardial infarction has never been fully implemented. It is possible that a fear of bleeding complications, the fact that later studies failed to demonstrate any effect of low fixed dose warfarin in addition to aspirin, and the need of monitoring of warfarin treatment have persuaded clinicians to opt for clopidogrel instead of warfarin as an addition to aspirin.

Risk factors, risk markers and determinants

There are very few conditions (exposures) that always lead to a particular outcome and in order to deal with this fact the term “risk” is often used. Risk is defined as the probability that an event will occur. The term “risk factor” is often used in medical research, but there are many uncertainties regarding its implications. There is no agreement as to whether a risk factor should be truly causal or only associated with an outcome and to what degree an association must be demonstrated in order to be conclusive. Likewise, there are also discrepancies as to whether a risk factor should be immutable or modifiable. Beck has suggested a definition of risk factor that has gained wide acceptance: “an environmental, behavioural, or biologic factor confirmed by temporal sequence in longitudinal studies, which if present directly increases the probability of a disease occurring, and if absent reduces the probability. Risk factors are part of the causal chain, or expose the host to the causal chain. Once disease occurs, removal of the risk factor may not result in a cure.” From this definition it follows that a risk factor per se does not have to be causal, rather only linked to the causal chain. The question of causality is complex, but some guidance can be found in the viewpoints published by Bradford Hill.

In Last’s A Dictionary of Epidemiology, a “risk marker” is defined as “an attribute or exposure that is associated with an increased probability of disease, but is not necessarily a causal factor.” A “determinant” is described as “an attribute or exposure that increases the probability of disease or other specified outcomes.” In this thesis, the use of the terms “risk factor”, “risk marker” and “determinant” refer to the above mentioned definitions.
Implications of bleeding complications

Besides the immediate effect and risks associated with a major bleeding event there also appears to be an association with long-term adverse outcome. The most obvious explanation for this association would be the fact that predictors of bleeding seem to be almost identical to predictors for ischemic events and, accordingly, that a bleeding event would act as a marker of increased risk of future ischemic events and mortality. There are other possible explanations as well. For example, anaemia in itself is known to be associated with adverse outcome in patients with acute coronary syndromes, and a sudden drop in hematocrit might induce hypoxia with subsequent maladaptive changes.

It is also common that anticoagulants and antithrombotic medication are discontinued during a bleeding episode and this might increase the propensity to thrombosis.

Finally, transfusion of whole blood could be a culprit in the observed increase in risk. It has been shown that transfusion in the absence of shock or hemodynamic instability is not consistently associated with a positive effect on survival. It is also known that transfusion could alter the immunologic response and the long term consequence of this is not fully understood. In general, a restrictive transfusion strategy is associated with a more favourable outcome.

In conclusion, there is evidence that bleeding complications carry significance not only on short term, but also on long term prognosis. It is likely that the event itself, either by inducing adaptive changes or by influencing treatment, is responsible for part of this increased risk in mortality. The identification of patients at high risk for bleeding complications are therefore of great importance.

Determinants of bleeding complications during oral anticoagulant treatment

When initiating vitamin K antagonist treatment it is important to take into consideration the risk of bleeding complications since a high risk profile could render the treatment with a net clinical effect that is negative. Linkins et al. performed a meta-analysis on studies involving patients with venous thromboembolism treated with oral anticoagulation which showed a high case fatality rate of 13.4% for major bleeding events indicating the seriousness of this complication. Compared to placebo, warfarin increased the risk of bleeding complications rather modestly, 1.0% compared to 1.3% in
patients with atrial fibrillation.\textsuperscript{106} However, the risk of intracranial bleedings have been shown to increase substantially with warfarin therapy with a relative risk of 10 for patients with warfarin treatment compared to the general population.\textsuperscript{107}

Evaluating the risk of bleeding in clinical practice is complex since there are several parameters to be considered. There is some evidence that the risk of bleeding complications varies with the treatment indication,\textsuperscript{108} most likely reflecting a varied patient population with different underlying risks. The incidence of serious bleeding complications vary a great deal between studies, ranging from as low as 2\% per year \textsuperscript{109} to approximately 13\%.\textsuperscript{110} A large proportion of this difference can be attributed to differences in the classification of bleedings. Furthermore, there seems to be a difference between patients on chronic treatment with warfarin and patients with newly initiated treatment since the risk of bleeding complications seems to be highest during the first months of treatment.\textsuperscript{111, 108}

**Age**

Clinical studies are often composed of a population with a relative low mean age \textsuperscript{112} which could explain why it has been difficult to identify advancing age as a risk factor for bleeding complications. Today an increasing body of evidence is gathering for age as a consistent risk factor for bleeding complication during oral anticoagulation.\textsuperscript{107, 113, 114, 115, 108, 116, 117}

**Previous gastrointestinal bleeding**

Previous bleeding from the gastrointestinal tract has been associated with an increased risk of bleeding during warfarin treatment in some studies.\textsuperscript{118, 119, 117} However, these results have not been definitively confirmed \textsuperscript{109, 120} and it is not clear how these results would be affected by modern eradication treatment of peptic ulcer disease.

Previous non-bleeding peptic ulcer has not been shown to be associated with future bleeding complications during oral anticoagulation.\textsuperscript{108, 121}

**Time in range**

The time in therapeutic range has been shown to correlate with both thromboembolic and bleeding events. Less time in therapeutic range is associated with an increased risk of both bleeding and thromboembolic events.\textsuperscript{116, 122} A more recent study have indicated that the variability in INR
INTRODUCTION

analyzed by calculating standard deviation of transformed INR is a better predictor, both of bleeding and mortality.\textsuperscript{123}

Gender

Studies have yielded diverging results regarding the bleeding risk attributable to gender. Earlier studies have found no association between gender and the risk of bleeding.\textsuperscript{114, 116} In a more recent study, Roldan et al. found that women had a higher risk of major bleeding compared to men in multivariate analysis.\textsuperscript{124} In a study of first time users of warfarin by Lindh et al., male gender was associated with an increased risk of severe bleeding events.\textsuperscript{125} As it stands today, no definite conclusion can be drawn regarding the risk of bleeding complications related to gender from the available data. This somewhat conflicting data needs to be investigated further since even a small increase in the risk of bleeding complication in women could affect the clinical benefit and potentially also affect the choice of anticoagulant treatment, especially in younger women with atrial fibrillation and no other risk factor for stroke.\textsuperscript{126, 127}

Renal function

A decrease in renal function is regarded as a risk factor for bleeding complications and is also stated as such in several guidelines.\textsuperscript{128, 129} The evidence for an association is weak \textsuperscript{109, 118, 119, 130} and it is only when kidney function is evaluated in combination with specific co-morbidities that a significant association has been shown.\textsuperscript{118} More recent studies have shown that a severe decrease in kidney function is associated with an increased risk of bleeding complications compared to patients with a mild to moderate decrease in kidney function, \textsuperscript{131} which in part could be explained by procedurally-related bleedings in the group of patients with a severe decrease in kidney function, at least for a sub-group with minor bleeding complications. In a systematic analysis from 2007 investigating dose-adjusted warfarin in patients with a severe decrease in renal function, \textsuperscript{132, 133} all studies lacked a comparison group with patients with normal renal function, so any comparison of bleeding events has to be made from historical data. In short, there is no prospective study comparing the risk with warfarin treatment with no treatment in patients with severely impaired renal function.

In the SPORTIF trial, which compared ximelagatran with warfarin in patients with atrial fibrillation, there was no significant association between a decrease in kidney function and risk of bleeding following multivariate analysis for patients with warfarin treatment.\textsuperscript{134} In a population with atrial
fibrillation, impaired kidney function (creatinine >200 µmol/l or a need for dialysis) was associated with an increased risk of bleeding. The incidence of bleeding was similar in patients with and without warfarin treatment, indicating that treatment with warfarin does not potentiate the risk associated with a decrease in kidney function. In that study the number of events was small and some caution when interpreting the data is therefore needed.

Studies investigating VKA treatment in patients with mildly to moderately decreased in kidney function have been equivocal. A recent study in patients with atrial fibrillation indicated that patients with a moderate decrease in eGFR (calculated by the Cockroft-Gault equation) were at a higher risk of bleeding complications, both during warfarin and rivaroxaban therapy. There was a substantial difference in age between the group with lower eGFR and the group with normal eGFR, namely 79 vs. 71 years. No adjustment for age was reported and thus it is not clear if the increased bleeding risk is simply an expression of increased age. A more recent study have indicated a substantial reduction in the risk of stroke without increased risk of bleedings for warfarin treatment in patients with stage 3 chronic kidney disease. Due to the low number of bleeding events these findings need confirmation.

In conclusion, the evidence for an increased risk of bleeding complications in patients with a mild to moderate decrease in renal function is sparse at best. Furthermore, it is not clear if treatment with VKA potentiates a pre-existing tendency to bleeding complications in this patient group.

VKA and concomitant pharmacotherapies

Aspirin increases the risk of bleeding in VKA-treated patients. In a meta-analysis, the relative risk of intracranial haemorrhage (ICH) when combining aspirin with warfarin was 2.4 compared to warfarin alone. Turpie et al. investigated the addition of 100 mg of aspirin to warfarin and noted a significant rise in the frequency of minor bleedings and a trend toward more major bleedings. The concomitant use of clopidogrel and aspirin is associated with a steep increase in the risk of bleeding.

Paracetamol is associated with an increase in INR values and one study has indicated that the incidence of gastrointestinal bleeding was higher in patients given paracetamol in combination with warfarin treatment compared to warfarin alone. No adjustment for confounders was made and it is possible that the intake of paracetamol is an expression of increased co-morbidity and, thereby, an increased risk of bleeding. In VKA-treated patients, non-steroidal anti-inflammatory drugs (NSAID) are associated with
an increased risk of gastrointestinal bleeding compared to warfarin treatment without NSAID.\textsuperscript{144}

Selective serotonin reuptake inhibitors have been shown to increase the risk of bleeding complications (HR 3.49) during warfarin treatment.\textsuperscript{145} These findings are supported in two retrospective studies.\textsuperscript{146, 147}

There are several other medications that interact with warfarin and necessitating a closer monitoring of INR values.\textsuperscript{148}

**Pharmacogenetics**

The CYP2C9 enzyme is responsible for the hydroxylation and metabolic clearance of the S-enantiomer of warfarin.\textsuperscript{149} There are 2 alleles with a lower dose requirement of warfarin and possibly an increased risk of bleeding complications, at least during the initiation of therapy.\textsuperscript{151, 152, 153} Vitamin K epoxide reductase (VKORC1) polymorphisms have been shown to be associated with lower dose needed to achieve a therapeutic INR value. This association is stronger than other related polymorphisms, such as those related to CYP2C9.\textsuperscript{154}

The identification of polymorphisms influencing dose requirements of warfarin could be of value before initiating therapy and it is possible that an implementation of genetic testing could reduce initial bleeding complications. It is reasonable to assume that in patients on long term VKA treatment, genetic polymorphisms are unlikely to affect the bleeding risk since the dose of warfarin is adjusted according to INR.

A much more rare mutation in factor IX is known to affect bleeding tendency in warfarin-treated patients not detected by standard INR measurements.\textsuperscript{155, 156} This is a result of a disproportional reduction in the levels of factor IX activity. Due to the low prevalence of the mutation it is not likely that it will affect routine healthcare.

**Other potential determinants of bleeding complications**

The presence of a malignancy is associated with an increase in the risk of bleeding complications.\textsuperscript{108, 157, 158} Some studies have shown an increased risk of bleeding after a previous ischemic stroke.\textsuperscript{115, 130} There are indications that a history of diabetes might be associated with an increased risk of bleedings, \textsuperscript{159} but a recent study did not show any significant association between history of diabetes and major bleeding complications.\textsuperscript{124} Hypertension has been associated with an increased risk of bleeding in multiple studies.\textsuperscript{160-162}
INTRODUCTION

Determinants of thromboembolic events in atrial fibrillation

Diabetes, hypertension, advanced age, previous stroke and congestive heart failure have all shown associations with an increased risk of stroke during atrial fibrillation. It has also been shown that patients with atrial fibrillation without previous stroke (or TIA), hypertension, diabetes and coronary heart disease have a similar risk of stroke as an age-matched population without atrial fibrillation, (1/100 patient years).

When applying these risk factors in risk stratification score such as CHADS$_2$, validation studies have shown an increasing risk of stroke for each additional risk factor present.

Declining renal function has also been suggested as an additional risk factor for stroke. In a cohort of patients with atrial fibrillation and no warfarin treatment, Go et al. noted that a lower eGFR was associated with an increased risk of thromboembolic events and that in combination with proteinuria this risk was enhanced independent of other risk factors. These findings indicate that a decrease in kidney function, measured as a calculated glomerular filtration rate, could complement risk stratification analysis in patients with atrial fibrillation.

Risk factors for cardiovascular disease

As early as the 1950’s, our understanding of risk factors responsible for the development of cardiovascular diseases began to grow. In a well known study from Framingham, three independent risk factors were described: weight, cholesterol and hypertension. In a more recent publication, the INTERHEART-study, it was concluded that most of the modifiable risk for cardiovascular disease could be explained by eight variables. The risk factors were: high blood lipids, smoking, hypertension, diabetes, obesity, psychosocial factors, a low consumption of fruit & vegetables, and alcohol, and, additionally, a lack of regular physical activity.

Risk factors for venous thromboembolism

Venous thrombosis is usually formed as a result of damage of the vessel wall, alterations in blood flow, or the presence of a hypercoagulable state. These factors are often referred to as Virchow’s triad and often accompany conditions which increase the risk of subsequent formation of venous thrombosis.
All types of surgery increases the risk of venous thrombosis, but patients that undergo orthopaedic, vascular or neurosurgery seem to be at an especially high risk. Immobilization and hospitalization for acute medical disorders confer an increase in the risk of venous thrombosis that is most pronounced for patients with myocardial infarction, respiratory failure and infectious diseases. For women, pregnancy and the period immediately postpartum are associated with a substantial increase in this risk, as is the use of oral contraceptives and hormone replacement therapy.

Trouseau was the first to recognize a connection between cancer and VTE with an increased risk of thrombosis in patients with malignancies. Conversely, it has also been noted that the incidence of malignancy is much higher in patients with a diagnosed VTE.

In addition to the risk factors already mentioned there are clinical conditions identifiable by biochemical markers, often referred to as thrombophilias. These include mutations in factor V, called factor V Leiden, mutations in factor II, G20210A, and deficiencies of antithrombin, protein C and protein S. Additionally, the presence of antibodies towards phospholipids, the antiphospholipid antibody syndrome, increases the risk of VTE.

Other conditions that have been associated with an increased risk of venous thrombosis include, among others, trauma, nephrotic syndrome, age, previous venous thromboembolism, the use of central venous catheters and high levels of factors VIII and XI. Recently, evidence indicating that risk factors for atherosclerotic diseases in general might be associated to VTE have been introduced and the presence of any one out of 33 autoimmune diseases has been reported to increase the risk of a pulmonary embolism.

**Risk stratification scores**

In order to estimate not only the risk of bleeding, but also the risk of thromboembolic events, several risk scores have been developed. The HEMORRHAGES and HAS-BLED scores are the most well known for estimating the risk of bleeding complications during VKA treatment. The clinical utility of the HEMORRHAGES score has been questioned since there is evidence that two-thirds of patients in an elderly population have similar risks for thromboembolic events (according to CHADS2) as for bleeding complications. The HAS-BLED score was developed and evaluated in patients with atrial fibrillation in the Euro Heart Survey cohort and SPORTIF study. Patients with higher values were at an increased risk of bleeding complications (see Table 1), yet, unfortunately, no concomitant
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data regarding incidence of thromboembolic events are available. The clinical usefulness of HAS-BLED is hampered by the fact that it is unknown if the net benefit of warfarin treatment in an individual patient outweighs the bleeding risk at a given score.

For the prediction of stroke risk, other scores have been developed. The most widely used is the CHADS\textsubscript{2} \cite{167} which is being replaced by the newly introduced CHA\textsubscript{2}DS\textsubscript{2}–VASc \cite{126,127} as summarized in Table 2. For each point increase in the score there is an increase in the risk of a future stroke and higher scores strengthen the indication for oral anticoagulation (see Table 1). An absolute cut-off value, identifying a high stroke risk, is not clear and it might be that this threshold exists at different points in different populations as indicated in a study by Hobbs et al. In elderly patients the risk scores seem to lose their ability to grade the risk of a future stroke and the predictive accuracy of the score were rather low.\cite{189}

Evidence is accumulating that a high CHADS\textsubscript{2} score might also be associated with an increased risk of bleeding complications and in a recent publication of more than 18,000 patients treated with warfarin or dabigatran it was shown that with increasing CHADS\textsubscript{2} score the risk of bleeding complications increased significantly.\cite{190} As seen in Table 2, the parameters for prediction of thromboembolic and hemorrhagic events share the same risk factors to some extent. For example, the additional risk factors of previous bleeding, renal impairment and labile INR have been associated with a higher risk of both hemorrhagic and thromboembolic events. Comparing hemorrhagic versus thromboembolic risk scores is not straightforward: the net clinical benefit of anticoagulant treatment subsides as the risk of bleeding complications predominates as demonstrated in Table 1. Some guidance could come from a recent retrospective population study which showed that the net benefit of anticoagulation compared to no anticoagulant treatment in patients with atrial fibrillation seem to be the highest in patients with a high CHADS\textsubscript{2} or CHA\textsubscript{2}DS\textsubscript{2}–VASc scores and a high HAS-BLED score.\cite{191} These findings indicate that the use of risk scores to evaluate the net clinical benefit is challenging. A high risk of stroke often goes hand-in-hand with a high risk of bleeding complications and that additional investigations with a prospective, randomized design and, perhaps the addition of biomarkers, are needed.
### Table 1. Risk of an adverse event according to risk-score

<table>
<thead>
<tr>
<th>Points</th>
<th>CHA$_2$DS$_2$VASc Stroke % / year</th>
<th>HAS-BLED Bleeding % / year</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>3.2</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>9.5</td>
</tr>
<tr>
<td>5</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>6</td>
<td>9.8</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>9.6</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>15.2</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 2. CHA$_2$DS$_2$VASc and HAS-BLED score, abbreviations and corresponding clinical characteristic.

<table>
<thead>
<tr>
<th>Point</th>
<th>Risk factor</th>
<th>Letter</th>
<th>Letter</th>
<th>Characteristic</th>
<th>Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Congestive heart failure/ LV dysfunction</td>
<td>C</td>
<td>H</td>
<td>Hypertension</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Hypertension</td>
<td>H</td>
<td>A</td>
<td>Abnormal liver or renal function</td>
<td>1 or 2</td>
</tr>
<tr>
<td>2</td>
<td>Age ≥ 75 years</td>
<td>A$_2$</td>
<td>S</td>
<td>Stroke</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Diabetes mellitus</td>
<td>D</td>
<td>B</td>
<td>Bleeding</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Stroke/TIA/Thromboembolism</td>
<td>S$_2$</td>
<td>L</td>
<td>Labile INR’s</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Vascular disease</td>
<td>V</td>
<td>E</td>
<td>Age &gt;65 years</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Age 65-74 years</td>
<td>A</td>
<td>D</td>
<td>Drugs or Alcohol</td>
<td>1 or 2</td>
</tr>
<tr>
<td>1</td>
<td>Female sex</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Biomarkers

The definition of a biomarker was standardized by a NIH working group in 2001 as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”. Biomarkers can be further classified depending on their intended use as antecedent, screening, diagnostic, staging, and prognostic biomarkers. Depending on the intended use of a biomarker the desired properties of a biomarker can vary. A high specificity, sensitivity, predictive value, and large likelihood ratio are important for a biomarker. When applying a biomarker in clinical medicine other factors such as the laboratory and biological variability need to be considered as well.

When looking for suitable biomarkers for thromboembolic and bleeding events during oral anticoagulation one needs to consider the mechanisms of not only oral anticoagulation, but also of stroke and cardiovascular diseases.

In general populations, multiple biomarkers have been investigated. CRP, VWF, fibrinogen, D-dimer, PAI-1 activity and factor VIIIc are all claimed to have a predictive value in addition to conventional clinical risk factors in the prediction of cardiovascular diseases. The predictive ability of different biomarkers is less well investigated in patients under VKA treatment.

Thrombomodulin

Thrombomodulin is a transmembrane glycoprotein expressed on the endothelial cell surface and it is also found in, thrombocytes and many other cell types. Thrombomodulin on the endothelial surface undergoes endocytosis and subsequent degradation and, to some extent, proteolytic release from the cell surface before later being secreted in the urine. Thrombomodulin present in plasma or urine is denoted soluble thrombomodulin (sTM) and has a half life of about 10-15 min in plasma before elimination by the kidneys. At least 7 distinct fragments compose sTM, each retaining enzymatic activity and the ability to activate protein C. There are indications that the distribution of fragments might differ between diseases. The expression of TM is transcriptionally up-regulated by several factors such as thrombin, vascular endothelial growth factor, histamine and statins. Hypoxia, TNF-α & IL-1β, transforming growth factor-β and oxidized LDL all have been found to downregulate TM expression. The biological roles of TM are divergent and impact coagulation, inflammation, and cell proliferation. Thrombomodulin plays an important role in both coagulation and fibrinolysis (see Figure 3). TM binds to
thrombin forming a complex that activates protein C, which in turn inactivates factors Va and VIIIa resulting in the inhibition of thrombin formation and an anticoagulant effect. In addition, thrombomodulin inhibits fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI). High levels of sTM have been suggested as a marker of endothelial cell dysfunction or damage. Elevated levels of sTM have been found in pre-eclampsia, lupus, diabetes, and disseminated intravascular coagulation. However, prospective studies have shown an increased risk for cardiovascular events both for low and high concentrations of sTM as well as an inverse relationship between levels of sTM and the risk of diabetes mellitus type 2.

A small, prospective cohort study reported that the number of serious bleeding episodes increased exponentially through quartiles of the sTM distribution. In addition, in a retrospective case-control study on VKA-treated patients a similar association was found for sTM.

The diurnal variation of thrombomodulin has been shown to be minimal which reduces the parameter of sampling time in epidemiological studies.

**von Willebrand factor**

Von Willebrand factor is a large, multimeric glycoprotein which mediates platelet adhesion, aggregation and also acts as a carrier for factor VIII. It is synthesized either in endothelial cells or megakaryocytes and stored as pro-VWF in Weibel–Palade (WP) bodies or alpha-granules. The rate of pro-VWF synthesis is known to be influenced by estrogens, corticosteroids and thyroid hormone. Pro-VWF is processed during the secretory pathway and becomes multimeric. VWF is released into the circulation either constitutively or in a regulated manner from WP bodies. Most of the circulating VWF consists of proteolytic fragments of larger multimers indicating that a regulated release from WP bodies is the dominant pathway for the release of VWF in to the circulation. The majority of the circulating VWF originates from endothelial cells and several substances such as desmopressin, epinephrine, adenosine, prostacyclin, vasopressin, as well as hypoxia and shear stress have been shown to increase the secretion of VWF in cultured endothelial cells.

Plasma levels of VWF are mainly regulated by genetic factors (75%) indicated by the close relationship with the ABO blood grouping where individuals with blood group O have the lowest values of VWF. Age, diabetes,
and inflammation are also associated with higher VWF levels in plasma.

The degradation of VWF occurs by proteolysis by the ADAMTS-13 protease. The clearance of VWF is much less well understood, but experiments on rats have indicated that clearance occurs primarily in the liver. Several studies have documented VWF's ability to predict future cardiovascular disease, both in healthy populations, as well as in a high risk population. The mechanism for this association is not clear, but VWF is regarded as a marker of endothelial damage and dysfunction which could indicate a possible role of VWF in the arteriosclerotic process, but this is contradicted by the finding that patients with severe von Willebrand disease do not have less intimal plaques than healthy controls.

In warfarin-treated patients, VWF seems to be associated with an increased risk of bleeding. Oddly, high levels of VWF seem to increase the risk of bleeding in VKA-treated patients.

D-dimer

D-dimer is a marker of fibrin degradation and its formation depends on three enzymes, thrombin, factor XIIIa, and plasmin. The D-dimer antigen remains undetected until it is revealed by the degradation of cross-linked fibrin by plasmin. D-dimer measured in plasma may be derived from soluble fibrin before incorporation into a fibrin clot or it may come from high molecular weight complexes released from an insoluble clot. Modern D-dimer assays use monoclonal antibodies to detect an epitope that is present on the factor XIIIa cross-linked fragment of fibrin, but not on fibrinogen or non-cross-linked fibrin degradation products and each assay has an unique specificity.

D-dimer has a well-established role in the exclusion of venous thromboembolism, both deep venous thrombosis and pulmonary embolism. It has also been proven to help predict the risk for recurrent venous thromboembolism.

Plasma levels of D-dimer are elevated in patients with atrial fibrillation not receiving warfarin and the introduction of warfarin normalizes these levels indicating that D-dimer could be a marker of thrombotic risk in patients with atrial fibrillation. Clinical studies have yielded conflicting results, but there are studies indicating that D-dimer is associated with cardiovascular events as well as with an increased risk of haemorrhage in patients with
atrial fibrillation and warfarin therapy. These findings were not confirmed by Roldan et al. In the Caerphilly Study, D-dimer was significantly associated with cardiovascular disease and ischemic stroke even when adjusting for conventional risk factors. Mahe et al. found a threshold value for D-dimer below which the risk of a cardiovascular event is very low (1.7% per year) in patients with pre-existing atrial fibrillation. The finding is somewhat difficult to interpret since anticoagulant treatment differed and the group with VKA therapy had significantly lower D-dimer levels than the groups not receiving oral anticoagulant and no stratification for VKA treatment was presented.

In conclusion, there is conflicting evidence regarding D-dimer as a predictor of ischemic and possibly hemorrhagic events in warfarin treated patients. The predictive value of D-dimer in patients with ongoing VKA therapy is not clear.

The complex between tPA and PAI-1

Tissue plasmin activator in plasma is largely bound to its inhibitors PAI-1, C1 inhibitor and antiplasmin and only a small percent of the unbound antigen represents in vivo activity.

Methods to measure the complexes of tPA have been developed and the complex between PAI-1 and tPA correlates strongly with tPA antigen in plasma as well as with PAI-1 activity. The complex between tPA and PAI-1 has been shown to predict re-infarction. A prospective, nested, case-control study from northern Sweden noted an association between the complex and the development of a first stroke.

A recent publication by Wennberg et al. concluded that after adjusting for inflammatory markers and other potential confounding variables, the tPA/PAI-1 complex was not significantly associated with myocardial infarction. To my knowledge no association between the tPA/PAI-1 complex and mortality has been found.

Tissue plasminogen activator

Tissue plasminogen activator (tPA) is a protease which cleaves plasminogen into plasmin and initiates fibrinolysis. The efficacy of tPA is greatly enhanced in the presence of fibrin. The majority of tPA in plasma is bound to its inhibitors (PAI-1 and antiplasmin, among others) and only a small fraction is present as free, active tPA. tPA is usually measured as tPA antigen which reflects the total amount of tPA, and is inversely correlated to tPA
activity. Accordingly, high levels of tPA antigen could reflect ineffective fibrinolysis.

In several studies, high levels of tPA have been associated with ischemic stroke as well as myocardial infarction. After adjusting for traditional risk factors, Smith et al. could not corroborate these findings since the relation between tPA and CVD or ischemic stroke was attenuated and non-significant after adjustment.

In patients on oral anticoagulant treatment, tPA has been associated with cardiovascular events and cardiovascular mortality.

**Cystatin C**

Cystatin C was discovered in the 1960’s and its presence in blood was confirmed by Grubb et al. Cystatin C belongs to a family of cysteine protease inhibitors which play an important role in intracellular metabolism. Cystatin C is produced by all nucleated cells. To some extent its production can be influenced by corticosteroids, thyroid hormones, inflammation, and muscle mass. The relationship between muscle mass and cystatin C is most likely due to the fact that muscle cells constitute the largest group of nucleated cells in the body. The impact of muscle mass on cystatin C is less than on creatinine. It is known that in people under 60 years of age, women have lower values of cystatin C than men and that cystatin C levels are affected by age. Levels are higher in people over 50-60 years of age.

Cystatin C is filtered freely in the glomeruli and is reabsorbed in the tubuli were it is catabolised and no circadian variation in cystatin C concentrations exists, making it suitable as a marker of GFR. As a marker of GFR, cystatin C has superior performance when compared to creatinine in the range between 60 and 79 mL/min per 1.73m². In some groups, such as paediatric populations, transplanted patients, diabetics, and the elderly, using cystatin C instead of creatinine could be advantageous.

Cystatin C has been studied as a biomarker in epidemiology. In a prospective study, Shlipak et al. showed that cystatin C is a predictor of death and cardiovascular events, a finding collaborated by Zethelius et al. in 2008. Other studies have proven cystatin C to be associated with mortality in patients with heart failure and ischemic heart disease.
Creatinine and eGFR

Creatinine is an amino acid derivative with a molecular mass of 113 kD and is the final product of creatinine phosphate catabolism. Creatinine in plasma is generated by skeletal muscle and it is also absorbed from digested food. It is filtered by the glomerulus and is also secreted by the proximal tubules. The clearance of creatinine thus exceeds the glomerular filtration rate. Since the level of creatinine in plasma is determined by its excretion and generation, the main determinants of the plasma concentration of creatinine are muscle mass and kidney function.

Other factors that can affect creatinine levels are medications that inhibit the secretion of creatinine without affecting GFR, for example trimethoprim. It is known that tubular secretion of creatinine varies among and within individual patients and in patients with low GFR the extrarenal secretion of creatinine can increase, mainly due to increased degradation in the intestine.

Levels of creatinine in plasma correlate weakly to all-cause mortality, but a more robust association has been found when the inverse relationship between the glomerular filtration rate and creatinine is used to calculate an estimated glomerular filtration rate (eGFR). There are several equations for calculating eGFR. Two of the most widely accepted are the Cockcroft-Gault and the Modification of Diet in Renal Disease (MDRD) equations. All equations have a common denominator in that they try to adjust for the inherent variation in creatinine values related to age, sex, racial group, and muscle mass. Both equations have been reported to be less accurate in populations without chronic kidney disease.

In a study from 2009, Go et al. noted that a lower eGFR, calculated by the MDRD equation, was significantly associated with an increased risk of thromboembolic events in patients with atrial fibrillation off warfarin treatment.

C-reactive protein

C-reactive protein is a well-studied acute phase reactant closely linked to inflammation. It is synthesised in the liver and is widely used in different clinical settings. CRP has a constant half-time and its concentration is only dependent on the rate of synthesis. Large prospective studies have demonstrated a clear association between CRP and cardiovascular disease, stroke, and mortality. A recent Mendelian randomization study indicates that genetic markers related to CRP concentration are not associated with
coronary heart disease and it follows that CRP in itself does not seem to contribute to the development of coronary heart disease, representing a risk marker rather than a risk factor.

In patients with atrial fibrillation, the levels of CRP add to the conventional CHADS\(_2\) risk score in predicting mortality. CRP levels are also correlated with the levels of haemostatic variables such as VWF.

**Storage of samples in epidemiological studies**

In epidemiological studies blood samples are often frozen and stored for some time before analysis is made. For fibrinolytic factors there are indications that a period of protracted preservation has negligible effects on the results of analysis, however changes in reagent kits could affect the results. Analysis is preferably made at the same time, with the same reagents for all subjects. For CRP and D-dimer, one study has indicated that prolonged storage time does not affect the stability of the tests. Long-term storage of cystatin C does not seem to introduce a significant variance.

Several different types of blood specimen collection tubes have been investigated: serum, EDTA plasma, citrated plasma, and Stabilyte plasma. Citrated plasma collection tubes is regarded as the golden standard for long-term storage in haemostatic factor projects.

In conclusion, there is evidence that storage of blood samples for future analysis does not introduce a significant bias when analysing the above-mentioned markers, especially if all samples are analyzed at the same time using the same reagent kits.
The objective of this thesis was to investigate if specific biomarkers can independently predict future bleeding complications, cardiovascular events, and mortality during long-term oral anticoagulant treatment.

The specific aims of this thesis were:

- to investigate the association between soluble thrombomodulin, and von Willebrand factor and the risk of bleeding complications and mortality as well as cardiovascular events.

- to investigate if renal function, reflected by creatinine, eGFR, and cystatin C, is associated with an increased risk of cardiovascular events, mortality or risk of bleeding complications.

- to investigate the association between factors of the fibrinolytic system and cardiovascular events, all-cause mortality, and bleeding complications.
MATERIAL AND METHODS

Study population

In 1996, the Umeå catchment area was inhabited by approximately 120,000 people and the Skellefteå catchment area by 80,000 people. Patients were recruited from the anticoagulation clinics at Umeå University Hospital and Skellefteå County Hospital. About 90% of patients with oral anticoagulation in the Skellefteå catchment area were monitored at the anticoagulation clinic; for Umeå the corresponding number was 80%. In the corresponding registers a total of 1,204 patients on oral anticoagulant treatment were identified, out of which 957 were considered to have a treatment indication for at least 3 months. All patients were invited to participate in our study by written invitation and given essential information. All 847 patients that gave their written consent were asked to donate blood samples at a planned INR testing. All participants had been treated with warfarin for at least 2 months prior to blood sampling.

A total of 11 patients died before blood sampling could be carried out, while 15 patients stopped their treatment. In 102 patients no study blood samples were collected at the initial INR testing. The final study cohort is described in Figure 5.

In total, 356 patients from the Skellefteå anticoagulation clinic were identified and asked to complete a questionnaire regarding hypertension, diabetes, self-reported weight and height, previous bleeding peptic ulcer, and previous peptic ulcer disease. INR values from the registers at the time of blood sampling were retrieved for the patients monitored at the Skellefteå clinic.
Figure 5. Study population as described in paper II.

Study design

The papers in this thesis are all based on a longitudinal cohort study where blood samples and clinical characteristics were collected before outcome events.
Follow-up

The date of blood sampling was set as the inclusion date. Patients were followed until event, cessation of anticoagulant treatment, migration, or until 12/31/2001. Events occurring within 5 days after cessation of anticoagulant treatment were included in the study as were events occurring more than 2 days after restarting anticoagulant treatment.

The hospital records for all patients admitted to hospital were reviewed for possible events. Records from the Department of Medicine, Cardiology, Surgery, Ear Nose and Throat, Ophthalmology, Urology, Neurology, Neurosurgery, and Orthopaedic surgery were reviewed. Possible events were recorded. Death certificates were obtained for all fatalities, regardless as to whether they occurred in hospital or not.

Classification of events

All events were registered and a panel of three experts reached consensus regarding classification. Major bleeding were classified according to Schulman et al., and encompassed fatal or symptomatic bleedings in a critical area or organ, i.e. intracranial, intraspinal, intraocular, retroperitoneal, intraarticular, or intramuscular, or bleedings causing a fall in haemoglobin level of 20 gL⁻¹ or more, or leading to transfusion of two or more units of whole blood or red cells. In addition, all bleedings that did not fulfill the criteria for major bleeding, but were considered to be the primary cause for admission to hospital or extended hospitalization, were registered. These bleeding events were combined with major bleedings into the variable clinically relevant bleedings.

Myocardial infarctions were classified according to MONICA criteria. A definite infarction fulfilled any of the following criteria:

- typical serial ECG progression, or
- elevated cardiac enzymes to more than twice the upper limit of normal in combination with typical symptoms and abnormal ECG changes, or
- elevated cardiac enzymes to more than twice the upper limit of normal in combination with a probable ECG changes and atypical symptoms.
MATERIAL AND METHODS

For fatal myocardial infarctions an autopsy finding consistent with recent myocardial infarction was also classified as myocardial infarction.

Stroke, both hemorrhagic and ischemic, was classified according to WHO criteria. An event was classified as a stroke if clinical signs of focal (or global) disturbances of cerebral function lasted more than 24 hours with no apparent cause other than a vascular origin. All transient ischemic attacks, silent brain infarctions, strokes caused by trauma, subdural haemorrhage, subarachnoidal haemorrhage, and acute stroke with concomitant brain tumour were excluded. A CT scan excluding an intracranial haemorrhage was needed for definite diagnosis of an ischemic stroke. Strokes that were judged to be primarily ischemic in origin with later hemorrhagic transformation were classified as ischemic strokes and not bleeding complications. All other intracranial bleedings were classified as a major bleeding complication.

Deep venous thrombosis and pulmonary embolism were classified according to findings on flebography, ultrasound, angiography, computer tomography, or autopsy. Rethrombosis was classified as an increase in the extent of the previously affected area in combination with symptoms. Peripheral arterial embolism required clinical symptoms plus objective verification either on angiography, at operation, or autopsy.

The cause of death was ascertained from death certificates. Deaths caused by stroke (both ischemic and hemorrhagic), intracranial haemorrhage, myocardial infarction, heart disease, or vascular diseases (i.e. aortic aneurysms) were classified as cardiovascular deaths.

Statistical analyses

Variables were tested for normal distribution with Q-Q plots; skewed variables were logarithmically transformed before being included in the analysis. All markers were analyzed both as continuous variables and as tertiles for comparing low, intermediate, and high levels and for detecting non-linear associations. For sTM, further investigations were made by dividing the variables in to quintiles and decentiles since no association with mortality was found for the continuous variable or tertiles. Spearman correlation coefficients were used to evaluate relationships between the variables.

The Mann-Whitney two independent samples test was used for continuous variables and the chi-square test for categorical variables when comparing baseline characteristics between the Umeå and Skellefteå cohorts.
MATERIAL AND METHODS

Time to event was analyzed using the Cox proportional hazard model. The assumption of proportional hazard was confirmed graphically using Kaplan-Meier survival curves.

All variables were included in a univariate Cox regression analysis and variables with a p-value of less than 0.20 were included in multivariate Cox regression models in order to estimate the effects of different determinants when controlling for covariates. A p-value of <0.05 (two-sided) was considered statistically significant. Individuals with missing values were excluded from the analysis.

With a statistical power of 80%, a hazard ratio (HR) of <0.44 or >2.0 was calculated to be significant at the 5% level for events with a prevalence of 2% per treatment year when comparing tertiles of biomarkers.

For continuous variables the results were presented as HR’s and 95% confidence intervals (CI) per increment in standard deviation for biochemical markers and per ten year increment for age. For tertiles the lowest tertile was used as reference. Direct age adjustment was made in ten year intervals for incidence of events.

Interaction analyses were made by dichotomizing the analyzed variables and then plotting the HR’s to check for interaction on the additive scale. We also checked for an interaction on the multiplicative scale where appropriate.

In order to ascertain which of the variables predicted each of the events independently, variables which showed significant associations in the multivariate analysis in their respective original study were included in a new multivariate model. Cox regression analysis with backward elimination was performed for each endpoint and variables with a p-value of >0.10 was eliminated in each step.

The predictive ability of the variables in this thesis was evaluated by calculation of predictive values and likelihood ratios. Predictive values were calculated for all events during the whole follow-up period.

Statistic analyses were made with SPSS software version 15-17 (SPSS Inc., Chicago, IL, USA)
MATERIAL AND METHODS

**Blood sampling**

Blood samples were collected at a planned INR check. Samples were drawn at baseline with a minimum of stasis and collected in standard siliconized, plasma tubes containing 0.13 mol/L sodium citrate. After centrifugation the plasma samples were frozen and stored at −70 C until analyzed.

**Laboratory procedures**

The inter-assay coefficients of variation (CV %) for individual analyses are presented in Table 3.

**Table 3.** Inter-assay coefficients for analysis performed in the different papers.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Interassay coefficient of variance in our hands, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombomodulin</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>4.1</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>3.6</td>
</tr>
<tr>
<td>D-dimer</td>
<td>7.3</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>5.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.0</td>
</tr>
<tr>
<td>tPA antigen</td>
<td>10.7</td>
</tr>
<tr>
<td>tPA/PAI-1 complex</td>
<td>6.3</td>
</tr>
</tbody>
</table>

CRP was analysed with an automated method (IMMULITE Diagnostic Products Corporation, Los Angeles, California). Von Willebrand factor was measured using ELISA (DAKO, Denmark) and presented as kUL⁻¹. Analysis of thrombomodulin was made by Ann-Kristin Öhlin at the Department of Clinical Chemistry, Malmö, by a method described by Öhlin et al. The analysis is an in-house ELISA method in which the antibodies sTM43b and sTM531 were used. John Morser at Berlex Biosciences, California, and the company PAION, Aachen, Germany, provided the antibodies.

The whole study population was analyzed at the same time and the laboratory staff was blinded to the event status.

**Measurement of fibrinolytic factors**

When measuring tPA one can measure free tPA (tPA activity) or tPA antigen which includes tPA bound to its inhibitors. Figure 6 illustrates the different
states of tPA in plasma. In paper four, tPA antigen which also is referred to as tPA mass concentration was measured.

**Figure 6.** Schematic figure of tPA presence in the circulation

The mass concentration of tPA and tPA/PAI-1 complex was determined with enzyme-linked immunosorbent assay (ELISA). The reagent kits used – Imulyse tPA (for tPA mass concentration) and TintElize tPA/PAI-1 (for tPA/PAI-1 complex mass concentration) – were purchased from Biopool. The method for measuring the tPA/PAI-1 complex is based on a 2-site ELISA which utilizes a polyclonal antibody toward tPA as a “catch antibody” and a horseradish peroxidase-conjugated monoclonal antibody toward PAI-1 for measuring purposes.

D-dimer was measured with an enhanced microlatex immunoassay method Sta-Liatest D-Di (Diagnostica Stago, Asnieres sur Seine, France).

**Measurement of markers of kidney function**

Creatinine was analysed on a Hitachii 911 multianalyser (Roche, Mannheim, Germany) with kits from Roche/Boehringer (Crea plus, enzymatic method). Cystatin C was measured by immunoparticle turbidimetry on a Hitachi 911 instrument with reagents from DAKO, Copenhagen, Denmark. Calculated glomerular filtration rate (eGFR) was estimated by the four variables Modification of Diet in Renal Disease (MDRD) Study equation. (GFR = 175 x standardized serum creatinine$^{-1.154}$ x age$^{-0.203}$ x 1.212 [if black] x 0.742 [if female]).
We also categorized eGFR into 4 groups reflecting either normal kidney function (>90 mL/min/1.73 m²), or mildly (61 to 90 mL/min/1.73 m²), moderately (30 to 60 mL/min/1.73 m²) or severely (< 30 mL/min/1.73 m²) reduced according to the Kidney Disease Outcome Quality Initiative of the National Kidney Foundation.316

**Ethical considerations**

The study complied with the Declaration of Helsinki and was approved by the Research Ethics Committee of Umeå University. All data analyses were performed on anonymous data sets without possibility of identification of individual patients and were made on personal computers without access to the Internet. All patients gave their written informed consent. Data was presented only on group level and the risk of violating the integrity of individual patients must be considered to be very low. Blood samples were drawn at the same time as a regular INR sampling and no extra venepuncture was required in the collection process. The additional risk added by the collection process was minimal. Patients were not informed about the results of our analyses.
RESULTS

Baseline

Baseline characteristic of the study population is presented in Table 4.

Table 4. Baseline characteristics

<table>
<thead>
<tr>
<th>Study cohort (n=719)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at inclusion, years (SD)</td>
<td>70 (11)</td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>268 (37)</td>
</tr>
<tr>
<td>Cystatin C , mg/L, mean (SD)</td>
<td>1.1 (0.4)</td>
</tr>
<tr>
<td>Creatinine, µmol/L, mean (SD)</td>
<td>84 (28)</td>
</tr>
<tr>
<td>Calculated glomerular filtration rate, mL/min/1.73 m2, mean (SD)</td>
<td>77 (21)</td>
</tr>
<tr>
<td>CRP, mg/L, mean (SD)</td>
<td>7.0 (14.9)</td>
</tr>
<tr>
<td>Thrombomodulin, mean ng/ml (SD)</td>
<td>6.1 (2.8)</td>
</tr>
<tr>
<td>Von Willebrand factor, mean kU L⁻¹ (SD)</td>
<td>1.61 (0.59)</td>
</tr>
<tr>
<td>tPA, ng/mL, mean (SD)</td>
<td>12.4 (5.6)</td>
</tr>
<tr>
<td>tPA/PAI-1 complex, ng/mL, mean (SD)</td>
<td>8.99 (5.40)</td>
</tr>
<tr>
<td>D-dimer, µgram/L, mean (SD)</td>
<td>0.28 (0.72)</td>
</tr>
<tr>
<td>Indications for OAC treatment, no. (%)</td>
<td></td>
</tr>
<tr>
<td>prosthetic heart valve</td>
<td>248 (35)</td>
</tr>
<tr>
<td>atrial fibrillation</td>
<td>228 (32)</td>
</tr>
<tr>
<td>venous thromboembolism</td>
<td>83 (11)</td>
</tr>
<tr>
<td>ischemic stroke</td>
<td>73 (10)</td>
</tr>
<tr>
<td>peripheral arterial thromboembolism</td>
<td>40 (6)</td>
</tr>
<tr>
<td>miscellaneous</td>
<td>40 (6)</td>
</tr>
<tr>
<td>not defined</td>
<td>7 (1)</td>
</tr>
</tbody>
</table>

There were fewer women (37%) than men. Mean follow-up time was 4.2 years and the maximum follow-up time was 5.6 years. The majority of patients at the Skellefteå anticoagulation clinic had a therapeutic INR at the time of blood sampling. In the whole population, prosthetic heart valve was
RESULTS

the most common indication for warfarin treatment, followed by atrial fibrillation. It is notable that baseline CRP was relatively high.

Data on baseline variables are presented for the entire study cohort. When comparing the two catchment areas, patients from Skellefteå were significantly older, had atrial fibrillation as indication for treatment significantly more often, and also had significantly higher values of D-dimer and cystatin C. For Umeå patients the presence of a prosthetic heart valve was a more common indication for treatment.

No other baseline characteristics differed significantly between the two catchment areas.

From the Skellefteå warfarin clinic there were additional baseline data available that are presented in Table 5. It is notable that INR was in therapeutic interval at the time of inclusion (baseline) in 86% of the cases.

**Table 5.** Additional baseline data available from the Skellefteå warfarin clinic.

<table>
<thead>
<tr>
<th>INR at the time of inclusion, %</th>
<th>n=356</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2.0</td>
<td>9</td>
</tr>
<tr>
<td>2.0-3.5</td>
<td>86</td>
</tr>
<tr>
<td>&gt;3.5</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Body-mass index, kg/m², mean (SD)</th>
<th>25.4 (3.8)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>History of</th>
<th>n=356</th>
</tr>
</thead>
<tbody>
<tr>
<td>hypertension, no. (%)‘</td>
<td>161 (45)</td>
</tr>
<tr>
<td>peptic ulcer, no. (%)‘</td>
<td>48 (14)</td>
</tr>
<tr>
<td>bleeding peptic ulcer, no. (%)‘</td>
<td>21 (6)</td>
</tr>
<tr>
<td>diabetes, no. (%)‘</td>
<td>48 (14)</td>
</tr>
</tbody>
</table>

**Bleeding events**

During follow-up 113 clinically relevant bleedings and 73 major bleedings occurred. The locations of the bleedings are presented in Table 6. Gastrointestinal and intracranial bleedings were the most common locations.
RESULTS

Table 6. Site of bleeding

<table>
<thead>
<tr>
<th>Site of bleeding</th>
<th>Clinically relevant bleeding</th>
<th>Major bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=113</td>
<td>n=73</td>
</tr>
<tr>
<td>Epitaxis</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Hematuria</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Aortic aneurysm</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>40</td>
<td>29</td>
</tr>
<tr>
<td>Intracranial</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Other*</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>

* perioperative, gynecologic, retroperitoneal, renal, pericardial tamponade, haemoptysis, hemorrhage, ocular.

In Table 7 the proportions of bleeding at different INR is presented. The majority of the bleedings occurred at a therapeutic level of INR (2.0-3.5). There were 11 fatal bleedings (0.4% per treatment year).

Table 7. INR at the time of bleeding events

<table>
<thead>
<tr>
<th>INR at time of event</th>
<th>Major no. (%)</th>
<th>Clinically relevant bleedings no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtherapeutic (&lt; 2.0)</td>
<td>9 (12)</td>
<td>15 (13)</td>
</tr>
<tr>
<td>Therapeutic (2.0 - 3.5)</td>
<td>44 (61)</td>
<td>73 (65)</td>
</tr>
<tr>
<td>Supratherapeutic (≥3.5)</td>
<td>20 (27)</td>
<td>25 (22)</td>
</tr>
</tbody>
</table>

Cardiovascular events and all-cause mortality

The occurrence of endpoints and the use of respective endpoints in the different papers of the thesis are shown in Table 8.
RESULTS

Table 8. Cardiovascular events and mortality endpoints in the thesis publications.

<table>
<thead>
<tr>
<th>Event</th>
<th>Paper 1</th>
<th>Paper 2</th>
<th>Paper 3</th>
<th>Paper 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality, n=161</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Cardiovascular mortality, n = 110</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Non-fatal cardiovascular events, n = 75</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Myocardial infarction, n = 47</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic stroke, n = 42</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

Non-fatal cardiovascular events were comprised of arterial embolisation, myocardial infarction, and ischemic stroke that caused hospitalization. A total of 75 non-fatal cardiovascular events occurred during follow-up; 36 patients had a myocardial infarction, 39 patients had a stroke, and 7 patients suffered an arterial embolus. Seven patients had both a myocardial infarction and a non-fatal stroke and were included in the non-fatal cardiovascular events group based on which occurred first.

Myocardial infarction and stroke, fatal or non-fatal, leading to hospitalization occurred in 47 and 42 patients, respectively. A total of 161 deaths were recorded during oral anticoagulation, of which cardiovascular death was registered as the cause in 110 patients.

Correlations

Correlations between biomarkers and age are presented in Table 9.
Table 9. Spearman rank order correlations between age and biomarkers in the study

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>CRP</th>
<th>VWF</th>
<th>D-dimer</th>
<th>tPA</th>
<th>tPA/PAI-1 complex</th>
<th>Thrombomodulin</th>
<th>Cystatin C</th>
<th>Creatinine</th>
<th>eGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.07</td>
<td>0.31**</td>
<td>0.2**</td>
<td>0.13**</td>
<td>0.07</td>
<td>0.23**</td>
<td>0.53**</td>
<td>0.17**</td>
<td>-0.36**</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td></td>
<td>0.29**</td>
<td>0.22**</td>
<td>0.22**</td>
<td>0.32**</td>
<td>0.15**</td>
<td>0.29**</td>
<td>0.08*</td>
<td>-0.16**</td>
<td></td>
</tr>
<tr>
<td>VWF</td>
<td></td>
<td></td>
<td>0.25**</td>
<td>0.32**</td>
<td>0.34**</td>
<td>0.31**</td>
<td>0.46**</td>
<td>0.23**</td>
<td>-0.31**</td>
<td></td>
</tr>
<tr>
<td>D-dimer</td>
<td></td>
<td></td>
<td></td>
<td>0.12**</td>
<td>0.09*</td>
<td>0.25**</td>
<td>0.28**</td>
<td>0.08*</td>
<td>-0.14**</td>
<td></td>
</tr>
<tr>
<td>tPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73**</td>
<td>0.03</td>
<td>0.26**</td>
<td>0.17**</td>
<td>-0.21**</td>
<td></td>
</tr>
<tr>
<td>tPA/PAI-1 complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1**</td>
<td>0.25**</td>
<td>0.13**</td>
<td>0.16**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.52**</td>
<td>0.31**</td>
<td>-0.32**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystatin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.55**</td>
<td>-0.71**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-0.81**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Result of the individual papers

In Table 10 and 11 the HR and 95% CI are presented for all the studied biomarkers in the different papers of the thesis.

Table 10. Univariate and multivariate analysis of biomarkers for major and clinically relevant bleedings. Results presented per increment in standard deviation for continuous variables. Hazard ratios and 95% confidence intervals.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Major bleeding</th>
<th>Clinically relevant bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>Creatinine, µ mol/L</td>
<td>0.90 (0.68-1.19)</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.76 (0.43-1.35)</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>0.94 (0.54-1.63)</td>
<td>-</td>
</tr>
<tr>
<td>Cystatin C, mg/L</td>
<td>1.28 (1.01-1.60)</td>
<td>1.08 (0.83-1.43)</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.92 (0.51-1.67)</td>
<td>0.69 (0.37-1.28)</td>
</tr>
<tr>
<td>High</td>
<td>1.60 (0.92-2.78)</td>
<td>1.06 (0.58-1.95)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.02 (0.81-1.28)</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.68 (0.37-1.23)</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>1.22 (0.72-2.08)</td>
<td>-</td>
</tr>
<tr>
<td>eGFR mL/min/1.73 m²</td>
<td>1.0 (0.99-1.01)</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.01 (0.57-1.78)</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>0.93 (0.52-1.65)</td>
<td>-</td>
</tr>
<tr>
<td>Age by 10 y</td>
<td>1.50 (1.17-1.93)</td>
<td>1.41 (1.07-1.86)</td>
</tr>
<tr>
<td>Sex</td>
<td>1.31 (0.82-2.08)</td>
<td>-</td>
</tr>
<tr>
<td>D-dimer, µgram/L</td>
<td>1.33 (1.06-1.67)</td>
<td>1.27 (1.01-1.60)</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.32 (0.72-2.42)</td>
<td>1.29 (0.70-2.36)</td>
</tr>
<tr>
<td>High</td>
<td>1.83 (1.04-3.24)</td>
<td>1.67 (0.94-2.97)</td>
</tr>
<tr>
<td>tPA, ng/mL</td>
<td>0.97 (0.77-1.22)</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.81 (0.46-1.44)</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>0.94 (0.55-1.64)</td>
<td>-</td>
</tr>
<tr>
<td>tPA/PAI-1 complex, ng/mL</td>
<td>1.05 (0.83-1.33)</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.03 (0.59-1.79)</td>
<td>-</td>
</tr>
<tr>
<td>High</td>
<td>0.97 (0.55-1.70)</td>
<td>-</td>
</tr>
<tr>
<td>VWF</td>
<td>1.33 (1.06-1.66)</td>
<td>1.22 (0.96-1.55)</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.49 (0.80-2.79)</td>
<td>1.32 (0.70-2.49)</td>
</tr>
<tr>
<td>High</td>
<td>2.53 (1.41-4.56)</td>
<td>2.1 (1.45-3.85)</td>
</tr>
<tr>
<td>sTM</td>
<td>1.44 (1.21-1.71)</td>
<td>1.39 (1.16-1.66)</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.99 (1.02-3.88)</td>
<td>1.82 (0.93-3.55)</td>
</tr>
<tr>
<td>High</td>
<td>2.69 (1.41-5.14)</td>
<td>2.33 (1.21-4.48)</td>
</tr>
</tbody>
</table>
**RESULTS**

**Table 11.** Univariate and multivariate analysis of biomarkers for cardiovascular and all-cause mortality and non fatal cardiovascular events. Results presented per increment in standard deviation for continuous variables. Hazard ratios and 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>Non fatal cardiovascular events n=75</th>
<th>Cardiovascular mortality n=110</th>
<th>All-cause mortality n=161</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Univariate</td>
</tr>
<tr>
<td>Creatinine, µ mol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.15 (0.91-1.46)</td>
<td>-1.17 (1.02-1.33)</td>
<td>1.14 (0.95-1.37)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.90 (0.60-1.33)</td>
<td>1.23 (0.75-2.00)</td>
<td>1.33 (0.81-2.17)</td>
</tr>
<tr>
<td>High</td>
<td>1.19 (0.68-2.10)</td>
<td>1.48 (0.92-2.39)</td>
<td>1.23 (0.82-1.85)</td>
</tr>
<tr>
<td>Cystatin C, mg/L</td>
<td>1.41 (1.13-1.78)</td>
<td>1.20 (0.88-1.42)</td>
<td>1.85 (1.55-2.15)</td>
</tr>
<tr>
<td>Low</td>
<td>1.55 (0.88-1.26)</td>
<td>1.65 (1.36-2.01)</td>
<td>1.69 (1.39-1.91)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2.42 (1.25-4.66)</td>
<td>2.34 (1.29-4.25)</td>
<td>1.86 (1.01-3.46)</td>
</tr>
<tr>
<td>High</td>
<td>3.32 (1.72-6.39)</td>
<td>4.94 (2.83-8.64)</td>
<td>3.43 (1.85-6.34)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.11 (0.89-1.39)</td>
<td>1.03 (0.97-1.10)</td>
<td>1.26 (1.05-1.51)</td>
</tr>
<tr>
<td>Low</td>
<td>1.17 (0.80-1.33)</td>
<td>1.14 (0.94-1.79)</td>
<td>1.37 (1.18-1.60)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.20 (0.66-2.10)</td>
<td>1.23 (0.74-2.50)</td>
<td>1.52 (0.99-2.28)</td>
</tr>
<tr>
<td>High</td>
<td>1.31 (0.74-2.31)</td>
<td>1.73 (1.09-2.75)</td>
<td>2.06 (1.38-3.06)</td>
</tr>
<tr>
<td>eGFR mL/min/1.73 m²</td>
<td>1.00 (0.98-1.01)</td>
<td>0.99 (0.98-1.01)</td>
<td>1.00 (0.98-1.01)</td>
</tr>
<tr>
<td>Age by 10 y</td>
<td>1.48 (1.16-1.89)</td>
<td>1.41 (1.06-1.88)</td>
<td>1.28 (1.02-1.61)</td>
</tr>
<tr>
<td>Sex</td>
<td>0.88 (0.50-1.55)</td>
<td>0.83 (0.53-1.30)</td>
<td>1.01 (0.54-1.99)</td>
</tr>
<tr>
<td>D-dimer, µgram/L</td>
<td>1.30 (1.02-1.65)</td>
<td>1.23 (0.96-1.56)</td>
<td>1.21 (0.99-1.47)</td>
</tr>
<tr>
<td>Low</td>
<td>0.84 (0.47-1.52)</td>
<td>0.83 (0.53-1.30)</td>
<td>1.01 (0.54-1.99)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.88 (0.47-1.52)</td>
<td>0.83 (0.53-1.30)</td>
<td>1.01 (0.54-1.99)</td>
</tr>
<tr>
<td>High</td>
<td>1.36 (1.09-1.73)</td>
<td>1.36 (1.06-1.73)</td>
<td>1.28 (1.02-1.61)</td>
</tr>
<tr>
<td>tPA, ng/mL</td>
<td>1.24 (0.98-1.56)</td>
<td>1.15 (0.91-1.47)</td>
<td>1.12 (0.93-1.35)</td>
</tr>
<tr>
<td>Low</td>
<td>1.24 (0.98-1.56)</td>
<td>1.15 (0.91-1.47)</td>
<td>1.12 (0.93-1.35)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.68 (0.94-3.00)</td>
<td>1.63 (0.97-2.91)</td>
<td>1.92 (0.73-4.04)</td>
</tr>
<tr>
<td>High</td>
<td>1.46 (0.80-2.68)</td>
<td>1.00 (0.71-2.40)</td>
<td>2.06 (1.29-3.77)</td>
</tr>
<tr>
<td>tPA/PAI-1 complex, ng/mL</td>
<td>1.37 (1.09-1.73)</td>
<td>1.36 (1.06-1.73)</td>
<td>1.11 (0.92-1.30)</td>
</tr>
<tr>
<td>Low</td>
<td>1.37 (1.09-1.73)</td>
<td>1.36 (1.06-1.73)</td>
<td>1.11 (0.92-1.30)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2.27 (1.21-4.29)</td>
<td>1.99 (1.05-3.78)</td>
<td>1.88 (0.88-2.19)</td>
</tr>
<tr>
<td>High</td>
<td>2.44 (1.30-4.60)</td>
<td>2.27 (1.21-4.28)</td>
<td>1.56 (0.72-1.38)</td>
</tr>
<tr>
<td>VWF</td>
<td>1.36 (1.09-1.73)</td>
<td>1.22 (0.96-1.55)</td>
<td>1.45 (1.19-1.79)</td>
</tr>
<tr>
<td>Low</td>
<td>1.36 (1.09-1.73)</td>
<td>1.22 (0.96-1.55)</td>
<td>1.45 (1.19-1.79)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.09 (0.61-1.94)</td>
<td>0.91 (0.51-1.64)</td>
<td>2.37 (1.35-4.83)</td>
</tr>
<tr>
<td>High</td>
<td>1.68 (0.97-2.91)</td>
<td>1.28 (0.73-2.56)</td>
<td>4.37 (2.59-7.85)</td>
</tr>
<tr>
<td>sTM</td>
<td>1.09 (0.51-2.33)</td>
<td>-1.20 (1.02-1.42)</td>
<td>1.06 (0.87-1.30)</td>
</tr>
<tr>
<td>Low</td>
<td>1.09 (0.51-2.33)</td>
<td>-1.20 (1.02-1.42)</td>
<td>1.06 (0.87-1.30)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.12 (0.64-1.94)</td>
<td>0.91 (0.51-1.64)</td>
<td>2.75 (1.69-4.46)</td>
</tr>
<tr>
<td>High</td>
<td>1.01 (0.57-1.71)</td>
<td>1.78 (1.09-2.91)</td>
<td>1.59 (1.06-2.4)</td>
</tr>
</tbody>
</table>
Thrombomodulin as a marker of bleeding complications during warfarin treatment (Paper I)

We investigated the association between soluble thrombomodulin and bleeding complications, as well as all-cause mortality and non-fatal cardiovascular events, during oral anticoagulation. Baseline values of sTM are shown in Table 4, page 37.

Thrombomodulin showed significant associations with an increased risk of bleeding complications, both for major and clinically relevant bleedings (see Table 10). For sTM as a continuous variable, the HR per increment in standard deviation was 1.39 (95% CI 1.20-1.60) for a major bleeding event. These results remained significant after adjustment for age and CRP in a multivariate analysis. For the patients recruited from the clinic in Skellefteå, INR values at the time of inclusion were available and demonstrated no significant relationship with measured sTM values or subsequent bleeding events.

There were no associations between sTM expressed either as a continuous variable or grouped in tertiles and all-cause mortality or cardiovascular mortality (see Table 11). In order to investigate a potential non-linear association, sTM was further divided into quintiles and decentiles without detection of any significant association.

Age was significantly associated to bleeding complications, mortality and non-fatal cardiovascular events. Stratification for age did not show any synergistic effects between age and sTM. For clinically relevant bleedings, stratification for sex showed that women with a sTM value in the highest tertile had a HR of 3.21 (95% CI 1.34-7.67) when compared to the lowest tertile. The age-adjusted incidence of clinically relevant bleeding for women in the highest tertile was 7.1 per 100 treatment years. Corresponding HR for men with a sTM value in the highest tertile was 1.85 (95% CI 0.95-3.61) compared to men with a sTM value in the lowest tertile. The age-adjusted incidence for men in the highest tertile of sTM was 5.0 per 100 treatment years. Age-adjusted incidence of major and clinically relevant bleedings in the study population is presented in Figure 7.

After excluding patients with a supratherapeutic value (INR>3.5) at the time of bleeding the results for sTM remained significant as a continuous variable (HR 1.32, 95% CI 1.11-1.57).

After additional adjustment for creatinine in the multivariate model sTM remained significantly associated with clinically relevant and major
RESULTS

bleedings. HR 1.36 (95% CI 1.17-1.57) and HR 1.44 (95% CI 1.21-1.70) per increment in standard deviation respectively.

Figure 7. Age-adjusted incidence of bleedings in tertiles of sTM.
Von Willebrand factor predicts major bleeding and mortality during oral anticoagulant treatment (Paper II)

The second publication investigated the association between VWF and risk of bleeding complications, cardiovascular mortality, and all-cause mortality. Baseline values for VWF are presented in Table 4. Survival plots for tertiles of VWF and different endpoints are shown in Figure 8.

VWF, as a continuous variable and as a tertile, was associated with both cardiovascular and all-cause mortality in the univariate Cox regression analysis. After adjustment for age, CRP, and creatinine in the multivariate analysis the associations remained significant.

VWF also showed an association with bleeding complications. In univariate analysis, VWF, expressed both as a continuous variable and the highest tertile, was associated to clinically relevant bleedings, as well as major bleedings. In multivariate analysis, however, only the highest tertile remained significantly associated to bleeding complications with a HR of 1.72 (95% CI 1.07-2.77) for clinically relevant bleedings, and a HR of 2.1 (95% CI 1.45-3.85) for major bleedings. For VWF as a continuous variable the association became attenuated and no longer significant.

The incidence of major bleeding complications in the highest tertile of VWF was 4.1 per 100 treatment years and 1.6 per 100 treatment year for the lowest tertile. Stratification for age showed that people over 70 years and with a VWF level in the third tertile had a HR of 6.6 (95% CI 3.8-11.7) for major bleeding complications compared to the rest of the population.
Figure 8. Survival plots illustrating proportions free of event in different tertiles of von Willebrand factor for major bleedings (A), clinically relevant bleedings (B), cardiovascular mortality (C), and all-cause mortality (D).
Cystatin C and creatinine as markers of bleeding and mortality during oral anticoagulant treatment (Paper III)

The purpose of this study was to investigate the relationships between selected markers of kidney function and both bleeding events and cardiovascular events. We chose to investigate the following markers: cystatin C, creatinine, and eGFR.

The baseline distributions of the different values are presented in Table 4.

None of the markers displayed any significant associations with bleeding complications in multivariate analysis. Cystatin C, creatinine, and eGFR were all associated to all-cause mortality in the univariate Cox regression analysis (see Table 11). In multivariate analysis only creatinine and cystatin C remained significantly associated to all-cause mortality. For cystatin C there was also an association to cardiovascular mortality. After adjusting for hypertension and diabetes (available from the Skellefteå clinic), the findings for cystatin C remained significant regarding the associations with all-cause and cardiovascular mortality. For non-fatal cardiovascular events only cystatin C demonstrated a significant association.

Figure 9 shows the age adjusted incidence of mortality in different tertiles of the different markers of kidney function. There is a substantial increase in the incidence of all-cause and cardiovascular mortality with increasing levels of cystatin C. No increase in the incidence of bleeding complications as the levels of cystatin C increases was seen.

When analyzing only patients with atrial fibrillation as indication for treatment the findings for cystatin C could be confirmed. The continuous variable of cystatin C showed a HR of 1.91 (95% CI 1.37-2.68) for all-cause and of 1.90 (95% CI 1.24-2.89) for cardiovascular mortality. The highest tertile of cystatin C, compared to the lowest, remained significantly associated with all-cause and cardiovascular mortality, HR 4.69 (95% CI 1.73-12.69) and HR 7.16 (95% CI 2.00-25.56), respectively.
**Figure 9.** Age adjusted incidence of all-cause mortality, cardiovascular mortality, and major bleedings in tertiles for markers of kidney function
RESULTS

Impact of fibrinolytic factors on bleeding, morbidity and mortality during warfarin treatment (Paper IV)

In the fourth paper of the thesis we investigated the different components of the fibrinolytic system in relation to cardiovascular events, mortality, and bleeding complications.

For major bleedings a significant association was seen for D-dimer, but not for the other variables as demonstrated in Table 10. After adjustment for age, hypertension, body mass index, and diabetes available from the Skellefteå clinic, D-dimer remained significantly associated with major bleedings, HR 1.43 (95% CI: 1.03-1.98). Neither tPA, tPA/PAI-1 complex, nor D-dimer were associated with clinically relevant bleedings.

This study also investigated the associations between fibrinolytic variables and myocardial infarction or ischemic stroke, including both fatal and non-fatal events. For myocardial infarction, D-dimer HR 1.37 (95% CI 1.02-1.84) and tPA/PAI-1 complex HR 1.56 (95% CI 1.15-2.13) as continuous variables showed significant associations in multivariate analysis. The highest tertile of tPA/PAI-1 complex also remained associated with myocardial infarction in the multivariate analysis HR 2.99 (95%CI 1.25-7.14). Only age was significantly associated with ischemic stroke in multivariate analysis.

A separate analysis was made for non-fatal cardiovascular events (ischemic stroke, myocardial infarction, and arterial emboli). For non-fatal cardiovascular events only tPA/PAI-1 complex remained associated in multivariate analysis.

Aside from age, only the highest tertile of D-dimer was significantly associated to all-cause and cardiovascular mortality.
RESULTS

General results (Papers I-IV)

Associations with all-cause mortality

The age-adjusted incidence of variables that were significantly associated to all-cause mortality in any of the papers are show in Figure 10.

![Figure 10](image)

**Figure 10.** Age-adjusted incidence of all-cause mortality per 100 treatment years in different tertiles for cystatin C, von Willebrand factor (VWF), C-reactive protein (CRP), and D-dimer.

Associations with bleeding events

As depicted in Figure 11, the age-adjusted incidence of major bleeding events in tertiles, for variables significantly associated with major bleeding events in multivariate analysis, are shown. sTM, VWF, and D-dimer are all associated with major bleeding events. sTM and VWF are also associated with clinically relevant bleedings.
Figure 11. Age-adjusted incidence of major bleeding per 100 treatment years in different tertiles for von Willebrand factor (VWF), D-dimer, and thrombomodulin.

**Backward elimination**

For each endpoint, backward elimination was performed for variables significantly associated with the outcome in each paper.

For all-cause and cardiovascular mortality, age, D-dimer, VWF, CRP, and cystatin C were included in the analysis. After stepwise elimination of non-significant variables (P<0.10), age, cystatin C, and VWF remained in the final model for all-cause and cardiovascular mortality. Age was the only variable that remained significantly associated with non-fatal cardiovascular events, while cystatin C and tPA/PAI-1 complex were eliminated.

For bleeding events, the associations for VWF and D-dimer became attenuated and no longer significant and only sTM and age remained significant in the final model.
Predictive value of the variables

Bleeding

Table 12 summarizes the positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LH+), and negative likelihood ratio (LH-) for sTM and VWF. Variables dichotomized at the low and the high tertiles.

Table 12. Predictive values and likelihood ratios for bleeding events during 4.2 years of follow-up. Variables dichotomized at the low and the high tertiles.

<table>
<thead>
<tr>
<th>Clinically relevant bleeding</th>
<th>PPV</th>
<th>NPV</th>
<th>LR+</th>
<th>LR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low vs intermediate/high</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>0.2</td>
<td>0.91</td>
<td>1.26</td>
<td>0.51</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>0.18</td>
<td>0.88</td>
<td>1.1</td>
<td>0.74</td>
</tr>
<tr>
<td>Low/intermediate vs high</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>0.22</td>
<td>0.87</td>
<td>1.87</td>
<td>0.43</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>0.2</td>
<td>0.86</td>
<td>1.31</td>
<td>0.56</td>
</tr>
<tr>
<td>Major bleeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low vs intermediate/high</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>0.12</td>
<td>0.94</td>
<td>1.24</td>
<td>0.53</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>0.14</td>
<td>0.92</td>
<td>1.72</td>
<td>0.66</td>
</tr>
<tr>
<td>Low/intermediate vs high</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>0.12</td>
<td>0.93</td>
<td>1.17</td>
<td>0.68</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>0.14</td>
<td>0.92</td>
<td>1.41</td>
<td>0.96</td>
</tr>
</tbody>
</table>

All-cause mortality

Biomarkers associated with all-cause mortality in Cox regression analysis (CRP, cystatin C, VWF, and D-dimer) were dichotomized at their median. The positive predictive value (PPV), negative predictive value (NPV), likelihood ratio positive (LH+), and likelihood ratio negative (LH-) were calculated and are presented in Table 13.
RESULTS

**Table 13.** Predictive values and likelihood ratios of CRP, VWF, D-dimer, and cystatin C for all-cause mortality during 4.2 years of follow-up. Variables are dichotomised at the median.

<table>
<thead>
<tr>
<th></th>
<th>PPV</th>
<th>NPV</th>
<th>LR+</th>
<th>LR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>0.48</td>
<td>0.43</td>
<td>1.19</td>
<td>0.83</td>
</tr>
<tr>
<td>VWF</td>
<td>0.45</td>
<td>0.32</td>
<td>1.44</td>
<td>0.6</td>
</tr>
<tr>
<td>D-dimer</td>
<td>0.43</td>
<td>0.41</td>
<td>1.37</td>
<td>0.72</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>0.42</td>
<td>0.3</td>
<td>1.7</td>
<td>0.52</td>
</tr>
</tbody>
</table>

**Combining biomarkers**

Based on the results of publications 1 and 2, we investigated if sTM could be of value in identifying patients at low risk of bleeding complications in a population at high or low risk of mortality as defined by VWF levels.

The population was stratified according to low or high VWF (dichotomized at median), Cox multivariate regression analysis showed that a sTM value in the lowest tertile of sTM was still significantly associated with a lower risk of clinically relevant bleeding than levels in the highest tertile of sTM (see Table 12). There was a similar trend for major bleedings, but significance was not reach, possibly due to lack of power.

In patients with low values of VWF, sTM did not distinguish between low risk patients to any significant degree as shown in Table 14 and Figure 13.

**Table 14.** Multivariate analysis for clinically relevant bleedings including age per 10 year increment interval and tertiles of thrombomodulin in patients with a VWF level above (n=359) and below (n=360) median. HR and 95% CI are presented.

<table>
<thead>
<tr>
<th></th>
<th>High VWF</th>
<th>Low VWF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High VWF</td>
<td>Low VWF</td>
</tr>
<tr>
<td>Age</td>
<td>1.4 (1.04 - 1.89)</td>
<td>1.46 (1.09-1.94)</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (n=78)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate (n=118)</td>
<td>2.19 (0.88-5.43)</td>
<td>1.74 (0.88-3.44)</td>
</tr>
<tr>
<td>High (n=144)</td>
<td>3.05 (1.28-7.30)</td>
<td>1.63 (0.77-3.45)</td>
</tr>
</tbody>
</table>
RESULTS

The incidence of clinically relevant bleeding, as well as all cause mortality are depicted in Figures 12 and 13. In patients with a VWF level above median the incidence of clinically relevant bleedings was 3.1 per 100 treatment years in patients with a low sTM value and 5.0 per 100 treatment years for patients with the highest sTM values. For patients with a VWF below median the incidence of clinically relevant bleedings was not consistently increased with higher tertiles of sTM.

**Figure 12.** Age-adjusted incidence of clinically relevant bleedings and all-cause mortality in tertiles of thrombomodulin in patients with a VWF level above median (n=359).
Figure 13. Age-adjusted incidence of clinically relevant bleedings and all-cause mortality in tertiles of thrombomodulin in patients with a VWF level below median (n=360)
DISCUSSION

General discussion

Methodological considerations

The study design in this thesis is a longitudinal cohort study. The cohort study has several advantages. Most importantly, cases and controls are derived from the same population and blood sampling was made before the occurrence of outcome events. This allows for estimations of event rates in exposure groups. In order to account for time at risk, Cox regression analysis was used.

It is important to reflect over the internal and external validity of the study in order to interpret the data and its transferability to clinical conditions.

Internal Validity

Assessment of exposure variables

A known diurnal variation exists for tPA and tPA/PAI-1 complex. Blood sampling was done in conjunction with a planned INR sampling that was carried out in a standardized fashion with the majority of sampling occurring in the morning, thus making this type of bias less likely. The interassay coefficients of the studied variables were all below 10% except for tPA (CV 10.7%). This is considered acceptable. All blood samples were analysed at the same time and with the same reagents in order to restrict the influence of various reagents kits. The laboratory staff that performed the analyses were blinded to the clinical outcome. The plasma samples were stored for 4 to 5 years. For tPA, tPA/PAI-1 complex, CRP, D-dimer, and cystatin C, we known that storage time introduces very little variance in the analyses. Sampling was obtained at a single time point, the findings must therefore be interpreted in the light of having only one measurement at baseline and the effects of changes during the entire observation period could not be examined.

The levels of VWF are significantly lower for persons with blood group O and this effect was not adjusted for. Von Willebrand’s disease occurs in about 1% of the population and is often associated with low levels of VWF. No patient with known von Willebrand’s disease was included in the study.
Confounders

Time in therapeutic range (TTR) is not adjusted for in the statistical models and we know that this variable can affect the risk of both bleeding and thromboembolic events.\(^3\) However, we have observed that a majority of bleedings occurred at therapeutic INR values and that, as a population, warfarin treated patients in Sweden have a high time in therapeutic range. An association between INR at sampling or event and the exposure variables could not be found. Only one patient was lost to follow-up due to migration and was followed until the date of migration.

Unfortunately, we only had access to data for potential confounders (specifically weight, height, hypertension, diabetes, peptic ulcer, and previous peptic ulcer disease) from the Skellefteå anticoagulation clinic. The results for the biomarkers were not affected when adjustment for these confounders was made. We did not adjust for the potential confounders of CHADS\(_2\), CHA\(_2\)DS\(_2\)VAS\(_C\) and HASBLED.

Evaluation of outcome

In order to find all non-fatal events, both bleeding-related and thromboembolic, we studied patient records from all episodes which required hospitalization regardless of the diagnosis. One investigator (ML) searched for events in hospital records in both Umeå and Skellefteå. All potential events were registered and later classified by three investigators that were blinded to the biochemical results. Thus, the risk of misclassification of non-fatal events is presumed to be low. We used well-defined criteria for all outcome measurements.

We used death certificates for classification of fatal events regardless of whether ancillary data was available from hospital records. This approach was used to insure that the classifications would not differ between patients that died at home and those that died in hospital.

In a separate study we evaluated different search strategies for finding bleeding complications. We searched the records for patients for a wide range of diagnoses related to bleeding. In doing so we were able to identify an additional 26% of major bleedings and an additional 23% of clinically relevant bleedings compared to a method using only diagnosis registries. For clinically relevant bleedings, this translates into a bleeding frequency per year of 2.9% for the method based on diagnosis registries versus 3.9% for the method used in this thesis. For major bleedings the corresponding numbers are 1.8% and 2.4%, respectively.
DISCUSSION

External validity

The cohort was recruited in a clinical population of warfarin-treated patients in two hospital-based clinics. The vast majority of the patients in the catchment area attended the clinics, making a selection bias unlikely. A high percentage of the invited VKA-treated patients were included; only 4.8 % of invited patients decline to participate. It is likely that the study population reflects a clinically representative sample of VKA-treated patients. There were no exclusion criteria other than not being on warfarin treatment for at least 2 months prior to enrollment and having a planned treatment of at least 3 additional months. Collection of blood samples was done prior to outcome endpoints and patients were followed-up prospectively.

Our finding of annual incidences of bleeding of 2.4% and 3.9% for major and clinically relevant bleeding events, respectively, are relatively low, but in the same range as that which has been reported previously.320

The demographics of present day warfarin-treated patients has changed compared to our study population mainly by an increase in the proportion of patients with atrial fibrillation as a treatment indication, and by an increase in age. We know that these trends could affect the risk of bleeding 108 and thromboembolic events and, as a result, we have conducted a subgroup analysis on patients with atrial fibrillation. The findings presented in this thesis are adjusted for age and the results for patients with atrial fibrillation were consistent with the results for the study cohort as a whole. This suggests that our results are likely to be applicable even to a contemporary population of warfarin-treated patients.

Although the proportion of warfarin-treated patients in the population has increased steadily in later years, the incidence of intracranial bleeding has been shown to be on the decrease.321 It is possible that the selection of patients suitable for warfarin treatment has improved, as well as the monitoring of treatment, and that the incidence of bleeding complications in today’s warfarin-treated population is lower than indicated in our study. However, a recent study on a warfarin-treated population in Sweden noted an incidence of bleeding complications that are in concordance with our findings.320
DISCUSSION

Individual papers

Thrombomodulin as a marker of bleeding complications during warfarin treatment (Paper I)

We found that soluble thrombomodulin was significantly associated with bleeding complications, both clinically relevant and major bleedings. There were no associations between sTM and mortality or non-fatal cardiovascular events. We also conducted a subgroup analysis on patients with atrial fibrillation with similar findings. When stratifying for gender it was notable that women had a higher HR for bleeding events than men. The reason for this difference is not clear and needs to be evaluated in future studies.

From previous studies we know that sTM is associated with an increased risk of bleeding events \(^{216,217}\) as well as mortality \(^{322}\) in patients with warfarin treatment and in patients without warfarin treatment.\(^{323,324}\) We could not find any association between sTM and mortality in our population.

Previous studies have given conflicting results regarding sTM and its relation to myocardial infarction. In a study by Salomaa, an inverse relationship to incident myocardial infarction was shown.\(^{212}\) No description of the method by which sTM was determined is given in the paper. Thögersen et al., in a nested, case-control study, noted that high values of sTM could be associated to an increased risk of developing a first-ever myocardial infarction during follow-up.\(^{213}\) It is notable that the results in this study were derived from a subgroup analysis on women, and that no such relationship was found for men. In a more recent cohort study by Morange et al., no association between sTM and the development of myocardial infarction or angina could be demonstrated.\(^{325}\) It is known that sTM is composed of several different fragments and the use of different antibodies in different sTM assays makes it difficult to compare results between studies. The evidence for a potential link between high levels of sTM and myocardial infarction is sparse and conflicting.

It is not clear if the high levels of measured sTM reflect an increase in expression, an increased proteolytic release of endothelial bound thrombomodulin, or a decreased clearance. When adjustment for kidney function was made by incorporating creatinine in the multivariate model, findings for sTM were not substantially affected indicating that a decreased renal clearance is not likely to explain the findings. Endothelial dysfunction as part of an inflammatory reaction is also contradicted by our findings as the data were unaffected by adjustment for CRP, as would be expected in that case.
sTM is a vital part of both coagulation and fibrinolysis and an increased expression of sTM on endothelial cells could alter the haemostatic balance mainly due to more extensive inhibition of coagulation via the protein C pathway. This would result in an increased risk of bleeding.

One also has to consider the possibility that the sTM measured in plasma is not indicative of an underlying process which affects haemostasis, but that the fragments of sTM in and of themselves affect coagulation, shifting the balance toward anticoagulation. From previous studies we know that fragments of sTM exhibit protein C activating potential. In our population with a relative low prevalence of bleeding events, it is likely that the negative predictive value of a low sTM value is the most useful clinical marker in that it identifies patients at a low risk of bleeding.

Von Willebrand factor predicts major bleeding and mortality during oral anticoagulant treatment (Paper II)

The main finding presented in the second publication is the association between VWF and cardiovascular mortality, all-cause mortality, as well as bleeding events during warfarin treatment. One previous study has indicated that high levels of VWF could be related to bleeding events during warfarin treatment and this could be confirmed in our study. In addition, Roldan et al. have published a study showing an association between high levels of VWF and cardiovascular events and bleeding events supporting our results.

VWF plays an important part in primary haemostasis and, intuitively, it is difficult to explain the finding of an association between a high level of VWF and an increased bleeding risk. Some guidance might come from the study by Vincentelli et al. which showed that low levels of the highest multimers of VWF can be present despite normal levels of VWF antigens and, in fact, could be related to an increased risk of bleeding complications. This finding is further strengthened by the fact that we know that the large multimers of VWF are necessary for an optimal haemostasis. In our study we measured VWF antigen and it is unclear to what extent this reflect the functional activity of VWF, but it is possible that an increase in antigen also reflects an increased proteolysis of larger multimeres, the main source of VWF in plasma in people without VKA treatment.

The connection between high levels of VWF and cardiovascular events and mortality is established. The mechanism is not entirely clear, but VWF is considered to be a marker of endothelial dysfunction and there is a known association between markers of inflammation, such as CRP, and
VWF. In patients with impaired kidney function, levels of VWF are known to be elevated and renal dysfunction is, in itself, associated with mortality and cardiovascular events. We adjusted for CRP and creatinine in this study without substantial alterations of hazard ratios, indicating that high levels of VWF are not merely an expression of inflammation or impaired kidney function.

The dual association of VWF with bleeding as well as cardiovascular events makes clinical application more difficult. There is also an indication that VWF could add to the predictive value of CHADS when predicting stroke risk in atrial fibrillation patients. VWF antigen appears hopeful as a marker for future cardiovascular events, but the clear association to bleeding complications makes it less useful.

Cystatin C and creatinine as markers of bleeding and mortality during oral anticoagulant treatment (Paper III)

In the third paper, cystatin C, creatinine, and eGFR were studied. Cystatin C was independently associated with all-cause and cardiovascular mortality, as well as non-fatal cardiovascular events. Cystatin C appeared more strongly associated to mortality than creatinine, for which only the association with all-cause mortality remained significant after adjustment for age. In the univariate analysis, eGFR showed similar associations as creatinine, but after adjustment for age in the multivariate analyses the associations with mortality became attenuated and non-significant.

The relationship between cystatin C and mortality or cardiovascular events is well known, but has not to my knowledge been shown in a population of VKA-treated patients. There are several ways in which cystatin C could be related to cardiovascular events. First, it is known that cystatin C is superior to creatinine in identifying patients with an early, mild decrease in kidney function and it is possible that the increased risk of vascular events can be attributed to kidney function. This is somewhat contradicted by the findings of Menon et al., who showed that cystatin C has a better predictive value for mortality than iohexol clearance, indicating that cystatin C reflects not just decreased kidney function. There is also a clear association between inflammatory markers and cystatin C, however the associations with cardiovascular events in our study and in previous studies remain even after adjustment for CRP in the multivariate analysis. Finally, it has been shown that cystatin C is associated with the number of coronary artery stenosis in patients with a GFR > 90 mL/min/1.73m². This could indicate that there is a direct effect of cystatin C on the vascular wall possibly related to its role as a protease inhibitor.
A new potential complication of warfarin therapy has been suggested recently, namely warfarin-related nephropathy (WRN). WRN is thought to occur in some patients when INR becomes supratherapeutic. In patients with chronic kidney disease an association between an INR above 3.0 and an increase in serum creatinine followed by a more rapid deterioration of kidney function has been seen. In a subset of patients that developed a rise in creatinine, renal biopsies have confirmed the presence of casts of red blood cells in the tubuli. In a retrospective study by Brodsky et al. in 2011, indications that WRN might also exist in patients without chronic kidney disease was noted. An intriguing explanation of the predictive ability of cystatin C for all-cause mortality and cardiovascular mortality is that cystatin C could identify a subset of warfarin-treated patients at high risk for deterioration of kidney function and death.

Although the mechanism for cystatin C and its association with cardiovascular events is not fully understood, it seems clear that cystatin C adds predictive value to both the measurement of kidney function and markers of inflammation in the prediction of cardiovascular events. The latter is also confirmed in our study.

We could not find any significant association between creatinine, eGFR, or cystatin C and bleeding complications. In published guidelines, decreased kidney function is regarded as a risk marker for bleeding complications during oral anticoagulation. There is little evidence for a linear association since most studies have failed to show any increased risk for bleeding complications in patients with mild to moderate decrease in kidney function. Roldan et al. noted an association between impaired kidney function and major bleeding events, but not clinically relevant bleeding events. The definition of impaired kidney function was not stated in the paper and it is therefore not possible to further interpret these results with regard to patients with mild to moderate renal impairment. It is notable that eGFR is often used to assess kidney function in cohort studies. eGFR is used to correct the inherent associations of creatinine with age, sex, and muscle mass. One study has indicated that the age and sex differences for creatinine persist after applying the MDRD algorithm and that the variance of eGFR values in the population is mainly determined by age differences. Age is consistently associated with both bleeding events and mortality and adjustment for age in multivariate analyses seems appropriate before evaluating the predictive value of eGFR. In clinical medicine, eGFR is often used to screen for impaired kidney function. New oral anticoagulants are excreted by the kidneys to different extents and a decrease in kidney function could lead to a prolonged half-time and an increase in concentrations. Increased concentrations of anticoagulants have the
potential to increase the risk of bleeding and since there is presently no available method to measure concentration of newer oral anticoagulants, their accumulation might be overlooked. It has been shown that estimation of GFR by the MDRD formula\textsuperscript{301} seems to underestimate GFR, while estimation by the Cockroft-Gault formula\textsuperscript{300} seems to overestimate GFR.\textsuperscript{295} Cystatin C is less affected by age, muscle mass, and sex when compared to creatinine. It is possible that a cystatin C-based GFR estimate would be more reliable in screening for impaired renal function before initiation of oral anticoagulation, especially in patients without known kidney impairment and with an eGFR between 60 and 90 mL/min/1.73m\textsuperscript{2}. Larger prospective studies are needed to evaluate which method of determining kidney function is preferable in patients with oral anticoagulant treatment.

In summary, our findings further underscore the superiority of cystatin C as a marker of cardiovascular risk as compared to creatinine or eGFR. The lack of an association between all three markers of kidney function and bleeding complications during warfarin treatment is important. Patients with a mild to moderate increase in markers of kidney function should be considered foremost to be at an increased risk of cardiovascular events, and not bleeding complications. A recent study indicates that in patients with atrial fibrillation and a moderate decrease in kidney function, use of adjusted-dose warfarin is effective in preventing stroke and does not increase the risk of bleeding complications when compared to aspirin or aspirin & low fixed dose warfarin. Some caution is prompted due to a limited number of bleeding events in both groups in this study.\textsuperscript{137}

For warfarin, continuous monitoring of INR offers an advantage in patients with decreased kidney function. For the newer anticoagulants it is not clear how to best monitor patients with mild to moderate renal impairment. This needs to be taken into consideration when initiating oral anticoagulant treatment.

**Impact of fibrinolytic factors on bleeding, morbidity and mortality during warfarin treatment (Paper IV)**

Fibrinolytic factors displayed different predictive profiles. The complex between tPA and PAI-1 was associated with both myocardial infarction and non-fatal cardiovascular events, while D-dimer was associated with mortality, major bleeding complications, and myocardial infarction. In multivariate analysis no significant association between tPA and the different outcomes were found.
Following idiopathic venous thromboembolism, D-dimer has been shown to predict a higher risk of recurrent venous thromboembolism in non-warfarin treated patients \(^{252}\) and, additionally, cardiovascular events and occult cancer.\(^{342}\) Previous studies regarding D-dimer in patients with oral anticoagulation have yielded conflicting results regarding D-dimer and risk for cardiovascular events in patients with VKA.\(^{124, 268}\) Our finding for D-dimer supports an association of D-dimer with adverse events during oral anticoagulation.

When interpreting the findings regarding D-dimer it is important to bear in mind that the levels of D-dimer have been shown to be affected by therapeutic warfarin treatment, in some cases resulting in normalization of D-dimer values after initiation of treatment.\(^{253, 268}\) This reduction of D-dimer seems to be dose-dependent since low dose warfarin (INR <1.2) did not reduce D-dimer levels.\(^{343, 344}\) A study of a Japanese population revealed that an INR of 1.5 to 2.0 was as effective as an INR above 2.0 in lowering D-dimer. It was also shown that D-dimer levels were significantly higher in older people and that a higher dose of warfarin might therefore be required to normalize their values.\(^{345}\) Mahé et al. have identified a group of patients with atrial fibrillation and D-dimer levels lower than 334 ng/ml as having an extremely low risk of cardiovascular events. The data presented in that study were not adjusted for age and other potential confounders, such as treatment. In the group with low D-dimer levels, the incidence of cardiovascular events was 1.9% compared to 7.6% in the group with elevated D-dimer levels, thus indicating that D-dimer could be of value when identifying patient at an increased risk of cardiovascular events. In the same study it was also noted that a rise in D-dimer during follow-up preceded a cardiovascular event. No data regarding levels of D-dimer and the risk of bleeding was presented.\(^{256}\)

Treatment with new oral anticoagulants has been associated with a reduction in D-dimer.\(^{346, 347}\) It is possible that measuring D-dimer could identify patients at increased risk of cardiovascular events irrespective of the type of oral anticoagulant treatment.

In warfarin-treated patients, the predictive value of tPA has only been evaluated in one previous study in which an association between tPA and mortality \(^{269}\) was found. This finding was not corroborated in the present study. A separate study has indicated that tPA levels are increased in patients with atrial fibrillation.\(^{348}\) It is not clear if the levels of tPA are affected by anticoagulant treatment, but withdrawal of apecoumarol did not increase the levels of tPA, thus indicating that tPA might not be affected by VKA treatment.\(^{349}\) After adjustment for age in the multivariate analysis, no
significant association between tPA and stroke could be seen in our population.

The tPA/PAI-1 complex is associated to an increased risk of stroke and recurrent myocardial infarction, as well as a first myocardial infarction, but has not previously been studied in a warfarin-treated population. We found an association between myocardial infarction and non-fatal cardiovascular events and the complex between tPA and PAI-1 in warfarin-treated patients. It is known that tPA/PAI-1 complex correlates with triglycerides and BMI and we did not adjust for these variables in this study. The results should be interpreted cautiously.

In conclusion, among the studied markers of fibrinolysis we found that D-dimer shows the best potential as a clinically useful marker of cardiovascular events in patients with oral anticoagulation.

**Limitations**

There are some limitations that need to be considered. Questionnaire data was only available from the Skellefteå warfarin clinic which hampered our ability to adjust for these risk factors. We did not have access to data on CHADS2, CHA2DS2VASc, or HASBLED, and the additive value of the biomarkers could not be determined. Socioeconomic factors, smoking and alcohol consumption were not adjusted for and the possibility of residual confounding needs to be considered.

Sampling was made at one occasion, which dilutes the precision of the biomarkers. It must be remembered that our findings are only applicable to a population on long-term oral anticoagulant treatment.

Due to a limited population size the power to detect associations is limited as described by the power calculation in the methods section. Absence of an association in our study should be interpreted with caution.

**Clinical utility**

The biomarkers studied in this thesis have different predictive abilities and the potential clinical utilization differs between them. sTM appears to have the potential to discriminate patients with a low risk of bleeding complications from patients with a high risk. One possible application of sTM measurement is as a complement to risk scores, for example in patients with a medium to high CHADS2 and a perceived increased bleeding risk where a low sTM level may argue for a net benefit of warfarin treatment.
VWF has already been tested in addition to risk scores and seems to add predictive power irrespective of score, both for bleeding and cardiovascular events. Measurement of VWF levels seem to be of somewhat limited value in clinical practice since patients with a high level will have a high risk of both cardiovascular events and bleeding complications. Analyzing VWF, therefore, will most likely not increase the likelihood of identifying patients with an increased net benefit of treatment.

Markers of kidney function, such as cystatin C, creatinine, and eGFR are in use in routine healthcare today. The findings in this thesis further emphasize that a mild to moderate decrease in kidney function, as measured by these markers, is more indicative of an increased risk of cardiovascular event rather than an increased risk of bleeding.

We found that cystatin C has a higher predictive potential as a marker of cardiovascular risk in patients with OAC when compared to creatinine. With the introduction of new anticoagulant treatments dependent on renal excretion, a large number of patients will need to be monitored regularly for signs of decline in kidney function, which may influence the net clinical benefit of treatment. eGFR, as estimated by MDRD algorithm, has recently been shown to classify 32% of patients into the wrong kidney function class according to the KDOQI chronic kidney disease classification when compared with glomerular filtration rate measured by $^{52}$Cr-EDTA clearance. In comparison with new oral anticoagulants, warfarin could be advantageous in patients with decreased kidney function due to its established monitoring procedures.

D-dimer is an established marker of venous thromboembolism. More and more evidence is gathering that the level of D-dimer is correlated to the level of anticoagulant effect achieved, irrespective of type of oral anticoagulant. Our findings indicate that D-dimer is a marker of the risk of cardiovascular events and bleedings.

**Future perspectives on biomarkers**

Today we know that estimating the risk of thromboembolic events and bleeding complications based solely on clinical risk scores is suboptimal. As a group, patients with a high risk of thromboembolic events are likely to have a net benefit of oral anticoagulant treatment although they also have an increased risk of bleeding complications. Biomarkers offer the possibility to further enhance detection of patients with a net clinical benefit of oral anticoagulant treatment.
Do biomarkers add to the predictive value of known risk scores such as HASBLED and CHA\textsubscript{2}-DS\textsubscript{2}-VASc?

The biomarkers we have investigated are far from ready for clinical application and more studies on specific biomarkers and their use in patients with oral anticoagulant treatment is needed. The findings in our cohort of warfarin-treated patients needs to be verified in other settings and several questions need to be addressed. Are the results valid for patients on treatment with new oral anticoagulants? Is the predictive ability of biomarkers different in patients planned for treatment than in patients on continuous treatment? Are levels of biomarkers affected by oral anticoagulant treatment? These questions can not be answered from the data in our study.

To address these concerns a prospective cohort study investigating biochemical markers in patients planned for treatment with OAC would be the next logical step in evaluating the utility of biomarkers. Blood samples should be taken prior to treatment, early after initiation of treatment, and also when patients are on chronic treatment with either warfarin or new oral anticoagulants. Adjustment for CHA\textsubscript{2}-DS\textsubscript{2}-VASc and HASBLED should be done.

Randomized controlled trials (RCT) are expensive and time consuming to undertake and when investigating new markers in patients with increased risk of adverse events there are several practical and ethical considerations to be made. Initiating new RCT’s solely to investigate biomarkers is therefore not easily carried out, but an intriguing possibility would be to use data from the large, randomized controlled trials comparing new oral anticoagulants with warfarin that have been conducted during the last few years.\textsuperscript{4,6,5} Analyzing biomarkers in this setting would most likely add to our current knowledge.

Evaluation of kidney function and D-dimer

Assessment of kidney function is of great importance in patients treated with new oral anticoagulants. Studies investigating how to evaluate and best monitor kidney function are needed. Due to the inability of present equations to estimate kidney function correctly it might be necessary to directly measure glomerular filtration rate in patients with eGFR below 60 mL/min/1.73 m\textsuperscript{2}. Cystatin C, being less influenced by sex, muscle mass, and other confounders, might have advantages when monitoring kidney function. Our current knowledge of the balance between the risk of bleedings
and the risks of thromboembolic events in patients with a mild to moderate decrease in kidney is limited. The addition of markers of kidney function both to risk scores for bleeding, as well as to risk scores for thromboembolic events, are needed to further clarify the optimal use of oral anticoagulants in this group of patients.

Further studies regarding D-dimer and the prediction of cardiovascular events are needed. One question that needs answering is if D-dimer, as a marker of endogenous fibrinolytic activity, can be used to determine patient response to anticoagulant treatment. It is also of importance to investigate if monitoring of D-dimer levels is of value in tailoring anticoagulant treatment. This research may even be extended to other preventive measures such as treatment of hypertension and hyperlipidemia.
CONCLUSIONS

In patients with long-term oral anticoagulant treatment:

- increased levels of thrombomodulin are associated with bleeding complications. No association between thrombomodulin and risk of cardiovascular events or mortality was found.

- Increased levels of von Willebrand factor is associated with cardiovascular events, mortality, and bleeding complications, irrespective of CRP levels and age.

- increased levels of cystatin C is associated with mortality and cardiovascular events, but not with bleeding complications.

- increased levels of creatinine are associated with all-cause mortality, but not cardiovascular events or bleeding complications.

- eGFR is not associated with mortality, cardiovascular events or bleeding complications.

- increased levels of D-dimer are associated with mortality, myocardial infarction, and bleeding complications.

- increased levels of tPA/PAI-1 complex are associated with a risk of myocardial infarction and non-fatal cardiovascular events, but not with mortality or bleeding complications.

- tPA levels are not associated with mortality, cardiovascular events, or bleeding complications.
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