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VENOUS THROMBOEMBOLISM IN WOMEN

RISK FACTORS AND LONG TERM FOLLOW-UP

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Stockholm 2013

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7549-247-6

TO ELIAS AND HANNA

ABSTRACT

Background
Venous thromboembolism (VTE) is a common and potentially life threatening disease. Women have different risk factors than men at both first and recurrent VTE. The aim of this thesis was to further identify and explore risk factors for VTE in young and middle aged women.

Methods
A nationwide case-control study of genetic and environmental risk factors for VTE in women aged 18-64 years was conducted in Sweden 2001-2009. The study was called Thrombo Embolism Hormone Study (TEHS) and comprised 1377 cases and 1402 age matched controls. In a sub study called hypo-TEHS, 244 of the cases included in the TEHS-study were followed up with plasma samples and questionnaires after cessation of anticoagulant therapy. Levels of coagulation factors and thrombin generation was measured and compared between obese and non obese patients. The women were also followed up at least 24 months after the diagnosis of VTE to assess data on recurrence. We investigated if there was any correlation between the levels of thrombin generation, resistance to activated protein C (APC) and increased risk of recurrent events. To assess the influence of other risk factors for recurrent VTE, all cases included in TEHS were followed up after four years in the TEHS-follow-up study.

Results
Data from the TEHS study presented in this thesis showed that family history of VTE was predictive of increased risk of hormone, surgery and cast related VTE in women. In the hypo-TEHS study we found that obese women with VTE had increased levels of thrombin generation with two different laboratory methods, compared to the non obese women with VTE. Obesity was also related to higher inflammatory markers such as CRP and fibrinogen. Furthermore we found that increased thrombin generation was associated to increased risk of recurrent VTE, as was APC-resistance in the absence of the factor V Leiden mutation. In the TEHS follow-up study the risk of recurrence in women was found to be only 2 %. When the risk of recurrence in patients with hormone related first thrombosis was evaluated we found it to be higher than in surgery provoked first event but lower than in women with unprovoked first thrombosis.

Conclusions
The most important risk factors for VTE in young and middle aged women are use of hormones, surgery and cast. Assessment of family history of venous thrombosis could help to better identify women at increased risk prior to hormonal treatment or decision on prophylaxis after surgery/ cast. Obesity is another important risk factor for both first and recurrent VTE. Obese female VTE patients had higher thrombin generation than non obese patients which could represent one mechanism of how obesity is related to the increased risk of recurrent VTE. In the clinical situation, the overall risk of recurrence in VTE must be weight against the risk of bleeding complications on anticoagulant therapy. Data from the hypo-TEHS study showed that women with APC-resistance in the absence of Factor V Leiden are at increased risk of recurrence. In the TEHS follow-up we fund the overall risk of recurrent VTE to be lower than the expected risk of bleeding complications. Integration of gender and knowledge on the specific risk factors in women when estimating the risk of recurrence could improve the care of VTE patients.

LIST OF PUBLICATIONS

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

I. **Sonnevi K**, Bergendal A, Adami J, Lärfars G, Kieler H
Self-reported family history in estimating the risk of hormone, surgery and cast related VTE in women.

Thrombosis Research 2013 June 22. (Epub ahead of print)

II. **Sonnevi K**, Tchaikovski SN, Holmstrom M, Antovic JP, Bremme K, Rosing J, Larfars G.
Obesity and thrombin-generation profiles in women with venous thromboembolism.

Blood Coagul Fibrinolysis 2013 Jul;24(5):547-553

III. **Sonnevi K**, Tchaikovski SN, Holmstrom M, Rosing J, Bremme K, Larfars G.
Thrombin generation and activated protein C resistance in the absence of factor V Leiden correlates with the risk of recurrent venous thromboembolism in women aged 18-65 years.

Thromb Haemost. 2011;106(5):901-7.

IV. Ljungqvist M, **Sonnevi K**, Holmström M, Kieler H, Lärfars G
Risk factors for recurrent venous thromboembolism in women.

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

NIH	National Institutes of Health
VTE	Venous Thromboembolism
aPTT	Activated Partial Thromboplastin Time
PT	Prothrombin Time
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
APC	Activated Protein C
DVT	Deep Vein Thrombosis
PE	Pulmonary Embolism
LMWH	Low Molecular weight Heparin
COC	Combined Oral Contraceptives
CHC	Combined Hormonal Contraceptives
HT	Menopausal Hormone Treatment
WHO	World Health Organisation
CT-scan	Computed Tomography Scan
TEHS	Thrombo Embolism Hormone Study
MPA	Medical Products Agency
KI	Karolinska Institutet
SNP	Single Nucleotide Polymorfism
CRP	C-Reactive Protein
CAT	Calibrated Automated Thrombography
ETP	Endogenous Thrombin Potential
TG	Thrombin Generation
OR	Odds Ratio
HR	Hazard Ratio
SD	Standard Deviation
BMI	Body Mass Index

1 INTRODUCTION

The human body consists of many complicated systems that maintain more functions than we can ever imagine. All these systems have to adjust to different circumstances throughout life with different challenges depending on the gender of the human body. One of the important systems that are influenced by gender is the coagulation system. Historically, most medical research has been performed by men on male subjects. Women were rarely included in clinical trials until just 20-30 years ago. In 1986 the National Institutes of Health (NIH) established a policy to include women in clinical research in an attempt to improve the situation. In 1990, the federal Government Accountability Office in the USA issued a report evaluating and criticizing the implementation and effectiveness of the 1986 NIH policy and the inclusion of women in clinical studies which caused public attention to the issue.

There are physical differences between women and men. Several diseases have proven to present with different symptoms and to be associated with different mortality depending on the gender of the patient. To be able to better diagnose and optimize treatment for all patients, there is a need to understand these differences. Venous thromboembolism (VTE) is a disease with different risk factors depending on if the patient is a man or a woman. Furthermore there is a difference in risk of recurrent events that is also associated to gender. Accordingly, we need to gain better knowledge on risk profiles, underlying mechanisms of venous thrombosis in patients of both genders. The focus of this thesis was to investigate risk factors and long term outcome of venous thromboembolism in young and middle aged women.

2 BACKGROUND

2.1 THE COAGULATION SYSTEM, AN OVERVIEW

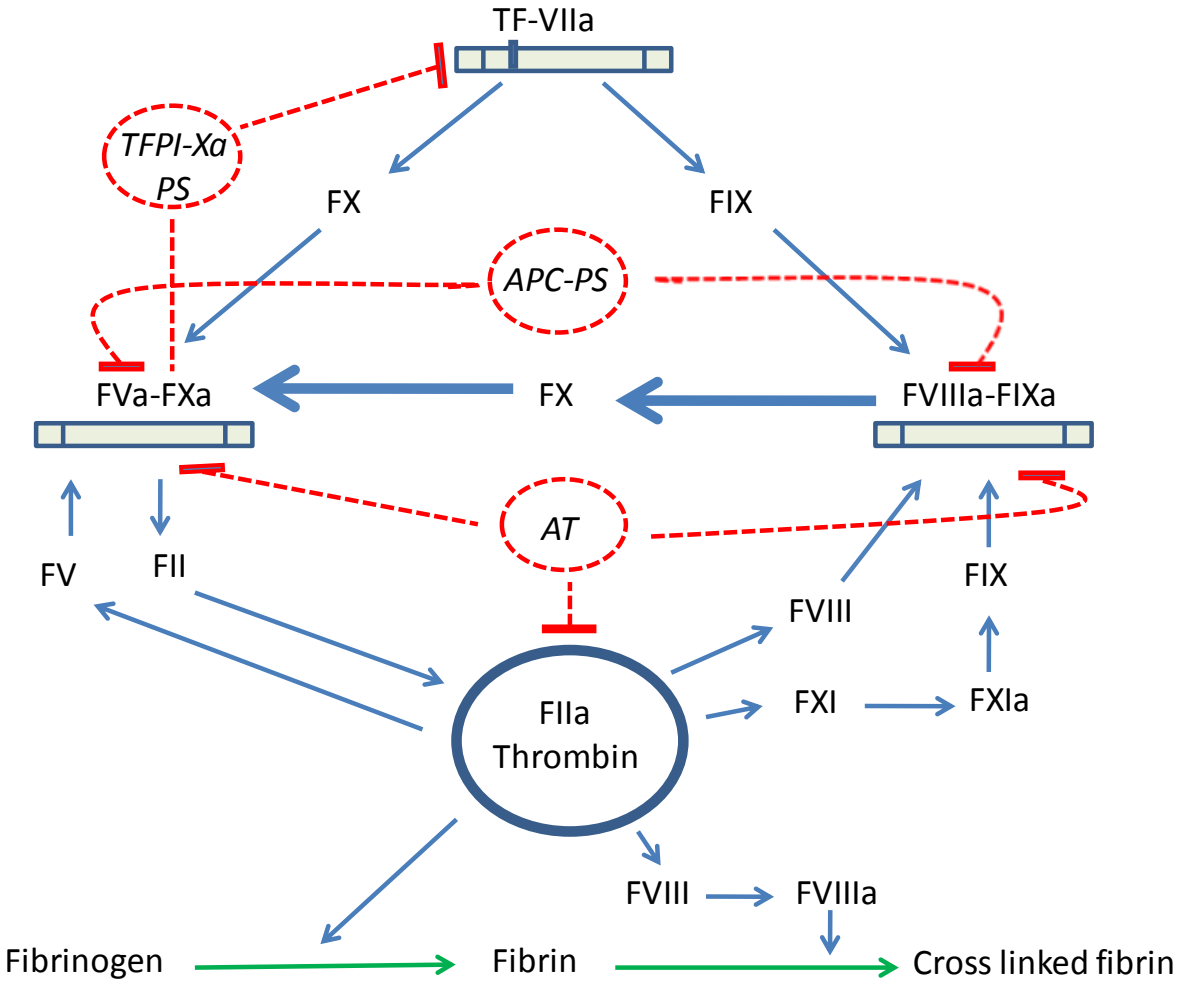
The circulation of blood in the human body is one of the basic conditions that must be met to keep us alive. Death can be caused by bleeding and blood loss, as well as uncontrolled formation of a thrombus in the arteries or veins, with cardiovascular death or pulmonary embolism as results. To prevent this, the bloods ability to clot is strictly regulated within the coagulation system. Haemostasis is a dynamic process that protects us from blood loss by rapid formation of a clot in response to tissue damage. At the same time the coagulation process is inhibited by a natural anticoagulant system to avoid hyper coagulation and thrombus formation. These systems are based on internal regulatory pathways to keep the haemostatic balance.
The three major components of importance in the haemostatic system are the platelets, the coagulation proteins and the endothelium (1, 2). These components act together to alter the blood flow only at the site of an injury to form a blood clot (3).

Platelets: When a vessel injury occur the exposure of the extracellular matrix will cause release of thrombogenic factors like endothelin and platelet activating factors. Platelets will adhere to the vessel wall and become activated. The activation of platelets leads to morphological changes including exposure of negatively charged phospholipids of the surface. Furthermore the platelets will release granules consisting of procoagulant proteins like factor V, factor VIII and fibrinogen. Other platelets are recruited to the site of injury to form platelet aggregates and the GPIIb/IIIa receptor is activated, facilitating platelet binding to cross linked fibrin.

The coagulation proteins: The classical description of the coagulation system is divided in an extrinsic and an intrinsic system both resulting in thrombin generation and ultimately in the formation of a fibrin clot (4, 5). The classical description of the coagulation system is useful in assessing imbalance in different coagulation tests such as the aPTT- and the PT-tests which are evaluating the intrinsic and the extrinsic pathway respectively. However, even if this description of the coagulation system is valid in vitro, it does not fully reflect how the coagulation system works in vivo.
The current explanation of how the coagulation system works in vivo was first published by Hoffman et al in 2001 (6). The proteins of the coagulation system are circulating in their inactive form and are activated through proteolysis. They then form active proteases or protease co-factors (factor Va, Factor VIIIa). Each activated protease can activate the next factor in a cascade like way. The initiation of coagulation begins with exposure of tissue factor (TF). An injury exposes TF, which can then bind to factor VII or factor VIIa to form a TF/ FVIIa complex (7-9). The TF/ FVIIa complex triggers activation of factor X to factor Xa. The TF/FVII complex can also activate factor IX, which binds to its co-factor VIIIa on negatively charged phospholipids and

then efficiently activate factor X to factor Xa. Factor Xa converts prothrombin (factor II) into thrombin (factor IIa) directly. More important, factor Xa and its co-factor, factor Va, forms a complex on the phospholipid surface called the "prothrombinase complex". This complex will greatly enhance the conversion of prothrombin to thrombin. Positive feed-back loops further enhances the thrombin generation by intrinsic activation of factor XI to XIa which in turn activates more factor IXa and factor VIIIa resulting in a burst of thrombin. The produced thrombin cleave fibrinogen to fibrin monomers which can crosslink in the presence of factor VIIIa to form a fibrin clot (3), figure 1.

Fig. 1



The endothelium and the regulatory systems of coagulation: The endothelium is, under normal conditions, designed to maintain blood fluidity in several ways. The regulatory systems of the coagulation cascade are largely dependent on the endothelium in the following ways:

1. Tissue factor pathway inhibitor (TFPI), an anticoagulant protease inhibitor, is secreted from the endothelium. TFPI forms a complex with factor Xa, which inhibits the TF/ factor VIIa complex. This results in down regulation of the TF-mediated thrombin generation, a process that is promoted by protein S (10, 11).

2. The endothelium is also a source of thrombomodulin, a cell surface receptor that binds to thrombin. When thrombin is bound to thrombomodulin it can activate protein C to activated protein C (APC). APC and its co-factor Protein S bound to phospholipids can inactivate factor Va and factor VIIIa and thereby down-regulate thrombin generation (12-14).

3. Antithrombin is a protease inhibitor present in plasma that binds to heparan sulphate present on the endothelium. The binding of antithrombin to heparan sulphate enhances the ability for antithrombin to inhibit thrombin 1000-fold (15, 16).

The balance between procoagulant systems and anticoagulant systems is essential to avoid bleeding or thrombosis. A prothrombotic phenotype is induced when the anticoagulant systems are down regulated or the sensitivity to anticoagulant systems is decreased (17, 18). This can also be achieved by enhancement of the procoagulant systems or, as in many cases, both. The procoagulant phenotype is related to increased risk of VTE (19, 20).

2.2 VENOUS THROMBOEMBOLISM

Venous thromboembolism includes deep vein thrombosis (DVT) and pulmonary embolism (PE). The recognition of the underlying mechanism of venous thrombosis in general, and pulmonary embolism in particular, was first published in 1856 by Dr R Virchow (21). He proposed an explanation to the main causes of thrombus formation that is known today as Virchows triad. The triad consists of stasis of blood, vessel damage, and hypercoagulability and this is still valid to some extent. Most described risk factors for initial and recurrent VTE are connected to one or more parts of the triad. VTE is a multi-factorial disease and several risk factors usually have to work synergistically to enable the formation of a thrombus and thereby causing DVT or PE (22). Most known risk factors influence two or more of the parts of this triad with hypercoagulability and the vessel wall being the most important factors (2, 23). Hypercoagulability occurs when there is an imbalance in the coagulation system between the procoagulant systems and the natural anticoagulant regulatory systems. In venous thrombosis a clot formation is often initiated after an acute or transient exposure to a risk factor. The clot might grow and may cause symptoms suddenly or, after a period of several days to weeks.

2.2.1 Epidemiology of VTE

Venous thrombosis is a disease affecting 0.5-2 / 1000 persons/ year (24-26). The most common sites of thrombosis are deep vein thrombosis of the leg or pelvis or pulmonary embolism (24). Less frequent sites include the portal vein, the splancnic vein, cerebral veins and the veins of the upper extremity. The incidence of VTE is slightly higher in men than in women, but at a younger age, women have a higher incidence probably due to pregnancy and female hormonal intake (27). Both men and women have an age dependent increasing risk of VTE but men seem to have a higher incidence than women after the age of 60 years (28-31). VTE is a typical multi-factorial disease with both genetic and acquired risk factors the affect the haemostatic balance (22, 32-35). Several risk factors for VTE have been identified and 50-75 % of all patients with a first event of VTE have at least one risk factor for VTE (25, 27) (29).

2.2.2 Risk Factors for VTE

2.2.2.1 Surgery and plaster cast treatment

Surgery and especially orthopaedic surgery of the lower limb is correlated with a high risk of postoperative VTE. The risk persists over at least a month after the surgical procedure (36, 37). Patients, who undergo major orthopedic surgery, are at a high risk of VTE. In this patient group, up to 40–60% of the patients have asymptomatic, objectively confirmed DVT (38). Research during the past ten years has shown that prophylactic treatment with low molecular weight heparin (LMWH) can reduce the incidence of postoperative VTE (38, 39). Patients undergoing general surgery such as abdominal, urological, gynaecological surgery have about 20 % risk of VTE whereas

the same kind of surgery in cancer patients is associated with a risk of almost 40%. Patients treated with plaster cast after a fracture of the lower limb or rupture of the Achilles tendon, are also at increased risk of VTE (40).

2.2.2.2 Combined hormonal treatment

Combined progestogen and estrogen, as combined hormonal contraceptives (CHC) and menopausal hormone treatment (HT), increase the risk of VTE in women (41, 42). The use of CHC is associated with a two- to six fold increase in risk of VTE in otherwise healthy young women (41-43). For HT, the risk is increased two- to four fold (44-47). The risk is mainly associated with the dose of estrogen and reductions of the concentration of ethinylestradiol from initial levels of > 50 µg to < 30 µg have reduced the risk of VTE significantly in CHC (48, 49). The same association with the dose of estrogen and risk of VTE have been suggested in HT, but is not as well established (50). In later years it has also become known that the risk of VTE in CHC-users varies with the sort of progestogen component. Desogestrel, which is used for the so called "third generation pill" is associated with higher risks of VTE than levonorgestrel or norgestrel, which are the progestogens used for the second generation pill (51-53). The underlying mechanism explaining the increased risk of CHC and HT has been investigated in several studies (54-57). Both CHC and HT have an influence on the coagulation system, mainly by causing an acquired resistance to activated protein C (APC) (58-60). Additionally, use of HT and CHC is associated with increased levels of procoagulants like prothrombin, FVII, FVIII, fibrinogen and decreased levels of natural anticoagulants like protein S, protein C and TFPI (61). The induced APC-resistance can work synergistically with inherited thrombophilias like the factor V Leiden and prothrombin gene mutation to cause clinically significant VTE in CHC and HT-users (62-64). Thrombophilia-screening of women prior to prescribing CHC or HT has been suggested but has not proven to be cost-effective and most current guidelines today do not recommend general screening (65). The use of CHC is associated with slightly higher relative risk estimates than HT, but the absolute risk of VTE associated with HT is probably higher since women who are treated with HT are older and have a greater burden of additional risk factors than women treated with CHC.

2.2.2.3 Progestogen only contraception

Progestogen only contraceptives (mini-pills) are not consistently associated with increased risk of VTE in studies available today (66, 67). Moreover, the levonorgestrel releasing intrauterine device has not been associated with an increased risk of VTE (43, 68) and is considered as a safe alternative for women with previous thrombosis.

2.2.2.4 Pregnancy

During pregnancy the haemostatic system changes towards a more procoagulant state with increased APC-resistance and lower levels of natural anticoagulants like protein S and C. There is also an increase in levels of coagulation factor V, VII, VIII, IX, XII and

d-dimer (69-71). These changes are protective of excess bleeding at the time of delivery and the haemostasis changes back to a normal status gradually post partum. Pregnancy is related to increased risk of VTE (72-74) with the highest incidence of VTE during the first three weeks post partum (75, 76). The most common site of VTE in pregnant women is DVT of the left leg or pelvic veins. This is due to anatomical conditions of the left iliacal vein and artery in combination with the pressure from the growing uterus (53).

2.2.2.5 Obesity

Obesity is an increasing medical problem in the world that is associated with increased risk of several chronic diseases and increased mortality (77). According to estimations by the World Health Organisation (WHO), 700 million adults in the world will be obese by the year 2015. There is consistent evidence that obesity is also correlated with increased risk of VTE (78, 79). Furthermore obesity is one of the few risk factors for first event of VTE that is also correlated with increased risk of recurrent VTE (80, 81). The mechanism of how obesity causes VTE is not fully understood but it is most likely a combination of stasis of the veins in the lower limbs, hypercoagulability related to low grade inflammation and other factors affecting the coagulation system (82). The correlation between low grade inflammation, thrombin generation and obesity has been reported to be more pronounced in women than in men, indicating a gender difference in how the haemostatic system is affected by obesity (47, 83, 84). In theory, the risk of VTE should be decreased by weight loss but there are currently no clinical studies that can confirm this. Bariatric surgery have shown to decrease the levels of thrombin generation in obese patients and weight loss programs have proven to reduce procoagulant changes in overweight children, indicating that weight loss could have an influence on the risk of VTE (85-87).

2.2.2.6 Family history of VTE

A positive family history of venous thrombosis has been used as a proxy for possible presence of inherited risk factors like factor V Leiden and the prothrombin gene mutation. Several studies have assessed the value of a positive family history in identifying patients with thrombophilias. In these studies the positive predictive value and sensitivity have been low, indicating that family history is of limited value in this regard (88-90). In recent years, a positive family history of VTE has emerged as a possible independent risk factor for VTE, regardless of the presence of any of the most common thrombophilias (91, 92).

2.2.2.7 Age

Age is one of the most important risk factors for a first event of VTE (30). Children and young adults rarely suffer from VTE. The incidence of VTE increase exponentially with increasing age with incidence numbers up to 1 per 100 patient-years in people above the age of 85 (27, 29, 31, 93, 94). Genetic and acquired risk factors seem to be as

important in the older population as it is in the younger even though the panorama of risk situations is different.

2.2.2.8 Cancer

Cancer is a well established risk factor for VTE and approximately 20 % of all fatal outcomes in VTE-cases are related to malignant diseases (25, 95). Patients with cancer have a 4-7 fold increased risk of VTE compared to patients without cancer. The incidence of cancer related VTE is 0.5-20 % depending of the type of cancer and occurrence of other risk factors (96, 97). Cancer of the brain and pancreas has the highest risk whereas breast cancer and prostate cancer patients are at lower risk of VTE (96, 98).

2.2.2.9 Genetic risk factors

Several inherited thrombophilias have been associated with an increased risk of VTE (99). Up to 25 % of all patients with a first event of VTE have a detectable inheritable thrombophilia which affect either the procoagulant or the anticoagulant pathways of the coagulation system. The most common inherited thrombophilias include the gain-of-function mutations factor V Leiden and prothrombin G20210A mutation. Deficiencies of antithrombin, protein C or protein S are other relatively common thrombophilias. The presence of one or more of the mentioned abnormalities is associated with increased risk of VTE (34, 100, 101). Thrombophilia testing in relatives to patients with known deficiencies of protein C, S or antithrombin may be useful to identify women who would benefit from prophylactic anticoagulant treatment during or after pregnancy (53, 102). Other than that, thrombophilia screening has not proven to affect the clinical management of patients and is not recommended to be performed on a routine basis in patients with VTE (103, 104).

2.2.2.10 Factor V Leiden

Factor V Leiden (R506Q) is a point mutation involving the factor V gene where arginine (R) at position 506 is replaced by Glutamine (Q). Normally, factor V is regulated by the natural anticoagulant APC (101). The R506Q-mutation is affecting the cleavage site for APC on factor V, reducing the ability for APC to inhibit Factor V. This results in APC-resistance and increased clotting tendency. The APC-resistance phenotype and its association with VTE was initially observed by Dahlbäck et al in 1993 and the site of the mutation was discovered and published by Bertina et al in Leiden in 1994 (105). Heterozygous factor V Leiden is associated with a moderate increase in risk of VTE whereas homozygous carrier ship is associated with higher risk estimates (79, 106-112). The heterozygous form of Factor V Leiden is prevalent in 3-8% of the Caucasian population with the highest prevalence of up to 15% in the Greek population (109, 110, 113-116). The mutation is however less prevalent in the Asian and African populations (117).

2.2.2.11 Prothrombin G20210A mutation

The prothrombin G20210A is a gain of function-mutation in the prothrombin gene causing increased levels of circulating prothrombin. The mutation was discovered and published in 1996 by Poort et al (100). Prothrombin G20210A is the second most common inherited thrombophilia in Caucasian populations with a prevalence of about 0.7-4.0 % (63, 118-123). Again, the prevalence of the mutation is low in Asian and African populations. The relative risk of VTE correlated with G20210A is two-five fold in heterozygous carriers of the mutation as compared to non carriers (112, 122-124).

2.2.3 Risk factors for recurrent VTE

Patients with a first episode of VTE associated with a provoking, transient risk factor are at low risk of recurrence (125, 126). Transient risk factors include surgery, trauma, plaster cast treatment and pregnancy. On the contrary, patients with a first VTE associated with a persisting provoking factor like advanced malignancy are at high risk of recurrence (126-129). Furthermore patients without an identified provoking risk factor are also at high risk of recurrence with 20-30% risk of recurrence in 5 years (130, 131). The duration of anticoagulant treatment beyond three months has not been correlated with the risk of recurrence (132). Accordingly, the risk of a new VTE is the same when the treatment is withheld regardless if it is after three months or three years. During the anticoagulant treatment period, there is however a very low risk of recurrent VTE. This means that patients at high risk of recurrence must be evaluated for infinite anticoagulation treatment. Infinite treatment is associated with substantial risk of bleeding and in some cases fatal bleeding complications. There is a need for good and validated systems to identify patients in whom the benefit from long term anticoagulation outweighs the risk of bleeding complications (133).

2.2.3.1 Gender

Men and women are at similar risk of developing a first episode of VTE but the risk of recurrence is different according to the gender of the patient. Men have a 1.5-2.5 fold higher risk of recurrent VTE and the reason for this is still largely unknown (81, 133-136). It has been suggested that the difference is due to a large proportion of women having their first episode provoked by CHC or HT. Theoretically it would be possible to remove the provoking factor of first VTE and thereby be at lower the risk of recurrence. To test this hypothesis some studies have re-evaluated their results concerning risk of recurrence in men and women without inclusion of women with a first hormone-associated VTE but the difference in risk by gender is still substantial (136).

2.2.3.2 Hormonal treatment

Patients with hormone associated VTE who resume their treatment with CHC or HT are at increased risk of recurrent VTE. The risk for women who stop using CHC or HT

after a first episode of VTE is less clear. There is an ongoing debate on whether hormone associated VTE should be considered as a provoked or non-provoked VTE (135, 137, 138). As the current guidelines advocate long-term anticoagulation in patients with unprovoked VTE it is of great importance to further clarify this (139).

2.2.3.3 Obesity

Obesity is well known risk factor for arterial thrombosis. In recent years several publications have confirmed that obesity is also a risk factor for both first and recurrent VTE (78-80, 82, 140-143). Obesity is associated with a two-three fold increase in risk of recurrence. Moreover, obese women have been described to have increased thrombin generation, indicating a hypercoagulable state related to obesity (84, 87, 144, 145).

2.2.3.4 Other risk factors

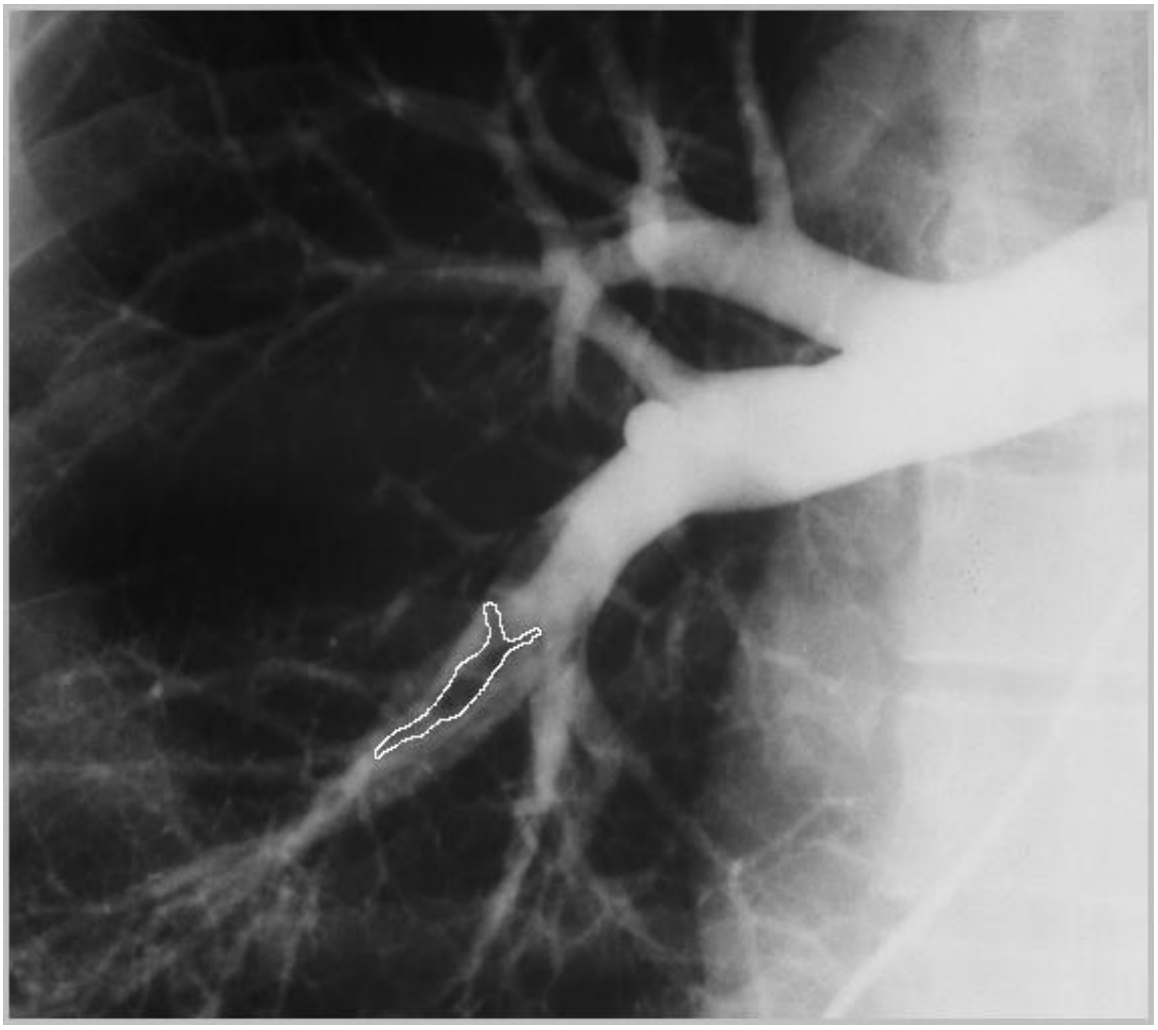
Several risk factors that are well established for the risk of a first episode of VTE are less important in the prediction of recurrence. For example, genetic thrombophilias like factor V Leiden and prothrombin G20210A do not show any substantial increase in risk of recurrent VTE (146-148). The same is valid for age, which is a strong predictor of first VTE but the reports on the influence of age on the risk of recurrence are conflicting (149-151).

2.2.3.5 Tests of hypercoagulability related to risk of recurrent VTE

Several global tests to estimate the coagulation have been proposed to be useful in predicting the risk of recurrent VTE. These tests include thrombin generation, different tests to assess the function of the protein C system and d-dimer. Many studies have evaluated the clinically available d-dimer test in predicting the risk of recurrent VTE with promising results (131, 152, 153). Several risk scores including testing of d-dimer during, or after cessation of, anticoagulant treatment have been useful in predicting high- or low- risk groups of patients (149, 154, 155). D-dimer is a degradation product of fibrin and is also influenced by inflammation and co-morbidity of the patient. Another available test is thrombin generation, a global test that estimates the overall coagulation status in a patient. Levels of thrombin generation have shown to be useful in discriminating between patients at high or low risk of recurrent VTE (156-160). The first thrombin generation test was introduced in 1953 (161). At that time, the assay involved sub sampling from the activated plasma mixture and quantification of thrombin in each subsample by measuring its ability to clot fibrinogen. Over time, fibrinogen was replaced by synthetic thrombin substrates, and sub sampling was replaced by continuous measurement of thrombin activity. Thrombin generation can now be measured in an automated way in a large number of plasma samples under different experimental conditions (60, 162). Tests of the protein-C pathway of the coagulation have also been reported to be useful in prediction of high risk of recurrent VTE. An example of such a test is the Protac-C assay (163).

2.2.4 Diagnosis of VTE

The diagnostic work up for VTE usually includes a combination of physical examination, laboratory testing and objective imaging techniques. The most validated diagnostic algorithm is based on Wells score (164, 165). The algorithm includes data on the patient's medical history, clinical signs of VTE and testing of d-dimer in case of low probability of VTE from the clinical scoring. A low score in combination with a normal d-dimer have a very high specificity in ruling out clinically significant VTE. In these cases no further testing is needed. In patients with high clinical scores or low scores with a positive d-dimer, further investigation with venography or duplex ultrasound of the limbs is recommended if the symptoms indicate DVT. If the clinical suspicion concerns PE the methods of choice are CT-scan, pulmonary artery angiography or perfusion/ventilation scintigraphy.



Pulmonary embolism

2.2.5 Treatment of VTE

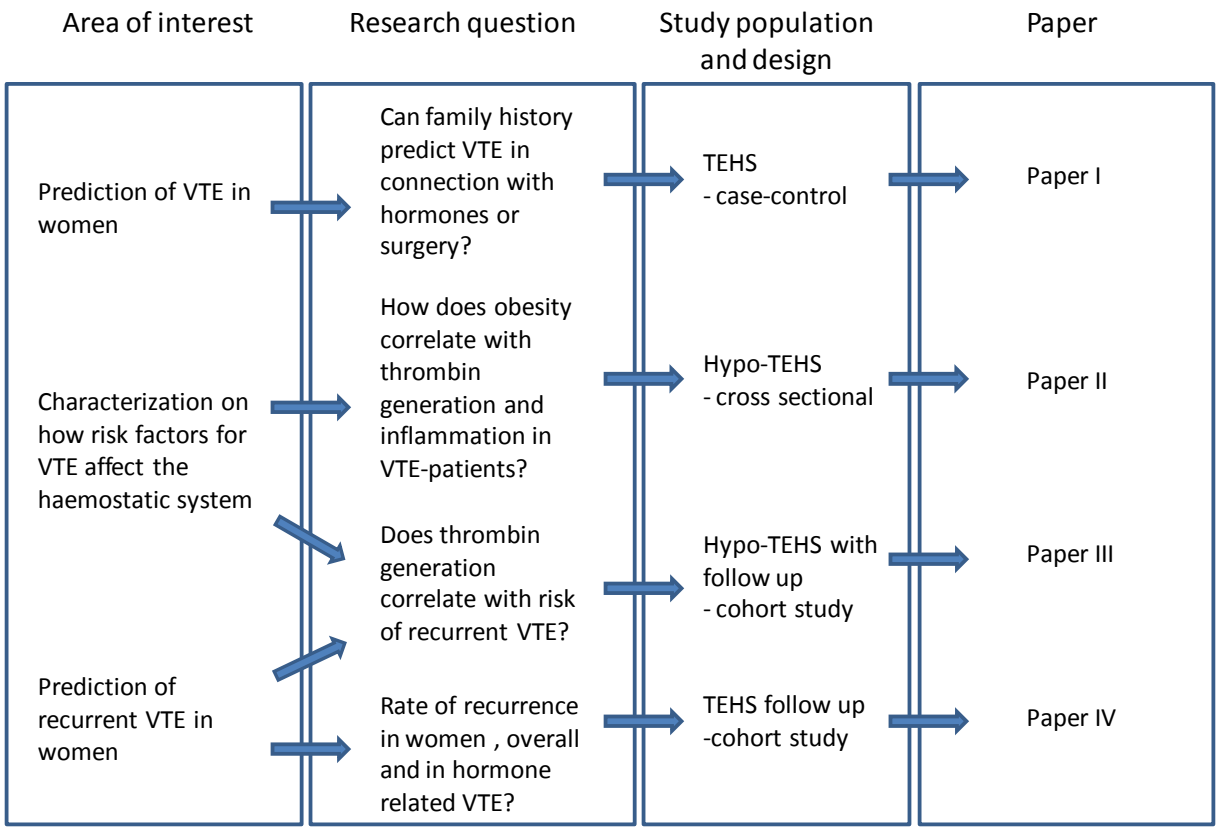
Treatment is usually initiated with subcutaneous injections with low molecular weight heparin (LMWH) and proceeds with warfarin when the diagnosis is confirmed. The treatment with LMWH is continued until the PT reaches therapeutic range (166). The duration of treatment is depending on the patients estimated risk of recurrence. As mentioned earlier an initial duration of 3 months treatment seems to be as effective as longer durations in terms of primary treatment. In high risk patients continuous anticoagulant treatment is recommended as secondary prophylaxis (139). Although warfarin is still the most used agent, new oral anticoagulants are now being launched. These agents act either as direct factor Xa inhibitors or as direct thrombin inhibitors and seems to be at least as effective as warfarin with the advantage of not needing constant monitoring (139, 167, 168). The risk of bleeding does not seem to be increased as compared to warfarin but the new anticoagulants are still lacking in specific reversal agents.

3 AIM OF THE THESIS

VTE is a common and potentially lethal condition with a multi-factorial etiology. The panorama of risk factors is different between men and women and also between first and recurrent VTE. The aim of this thesis was to further identify and explore the risk factors of VTE in young and middle aged women. To do this we conducted clinical and mechanistic studies on women 18-64 years with the following specific aims:

1. To estimate the risk of a hormone- or surgery/ cast related first VTE in women with and without a positive family history of VTE.
2. To explore the association between obesity, thrombin generation and inflammation in women with VTE.
3. To study if the risk of recurrence in women was related to increased thrombin generation and resistance to activated protein C in the absence of Factor V Leiden.
4. To study the overall risk of recurrence in young and middle aged women and estimate the risk associated with use of CHC/ HT compared to idiopathic and surgery/ cast related first VTE.

3.1 OVERVIEW OF THE THESIS



4 METHODS

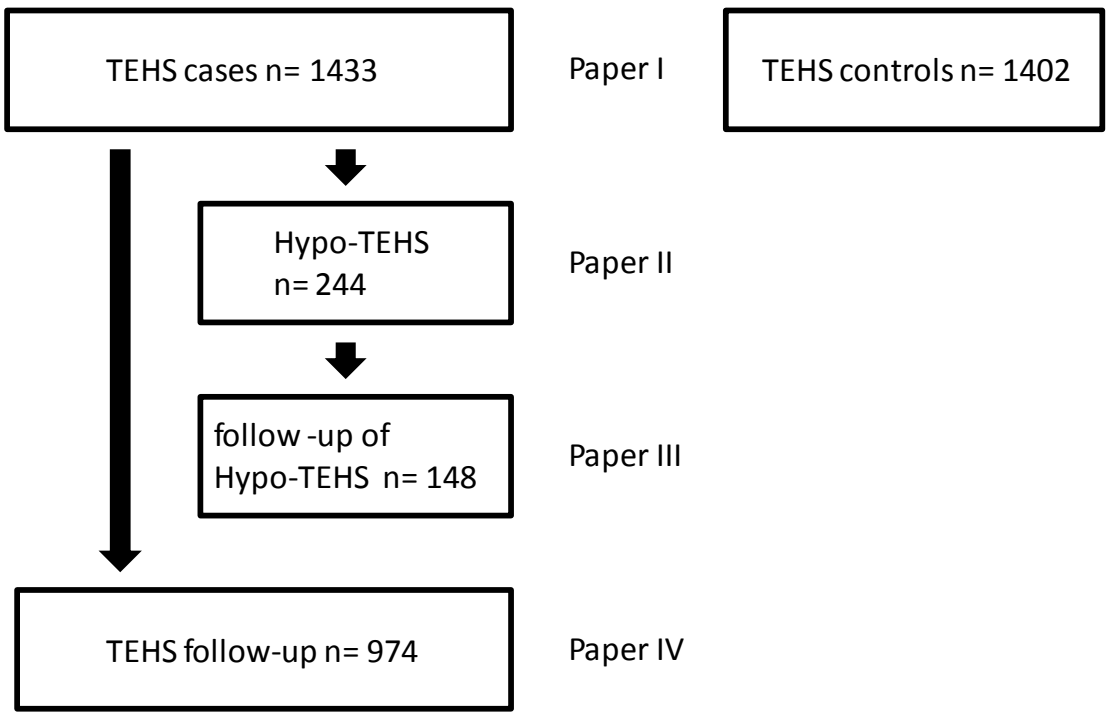
4.1 ETHICAL CONSIDERATIONS

All studies were approved by the regional ethics committees in Sweden and the participants signed an informed consent after oral information about the study. The women were informed that they could leave the study at any point and that their stored information and blood samples would then be destroyed.

4.2 STUDY POPULATIONS

The study-objects of this thesis were included as either cases or controls in the Swedish, nationwide case-control study "Thrombo Embolism Hormone Study" (TEHS). The TEHS-study was initiated from the Swedish Medical Products Agency (MPA) in collaboration with the Karolinska Institute (KI) and the Royal Institute of Technology (KTH). To be able to perform mechanistic studies and further explore the changes in coagulation factors and other haemostatic variables in women with VTE, a sub study comprising 244 of the TEHS-cases included in Stockholm, Linköping and Gothenburg was performed. This study was called "hypo-TEHS". The women in hypo-TEHS were followed-up at six months after their first VTE and also after 24 months and at time of closing the study in 2009 to assess recurrence. Finally, a follow up regarding recurrence and mortality of all 1433 cases in THES was conducted in 2011 in the" TEHS-follow-up" study. A schematic picture of the study-populations and how they are related is presented in figure 2.

Fig. 2



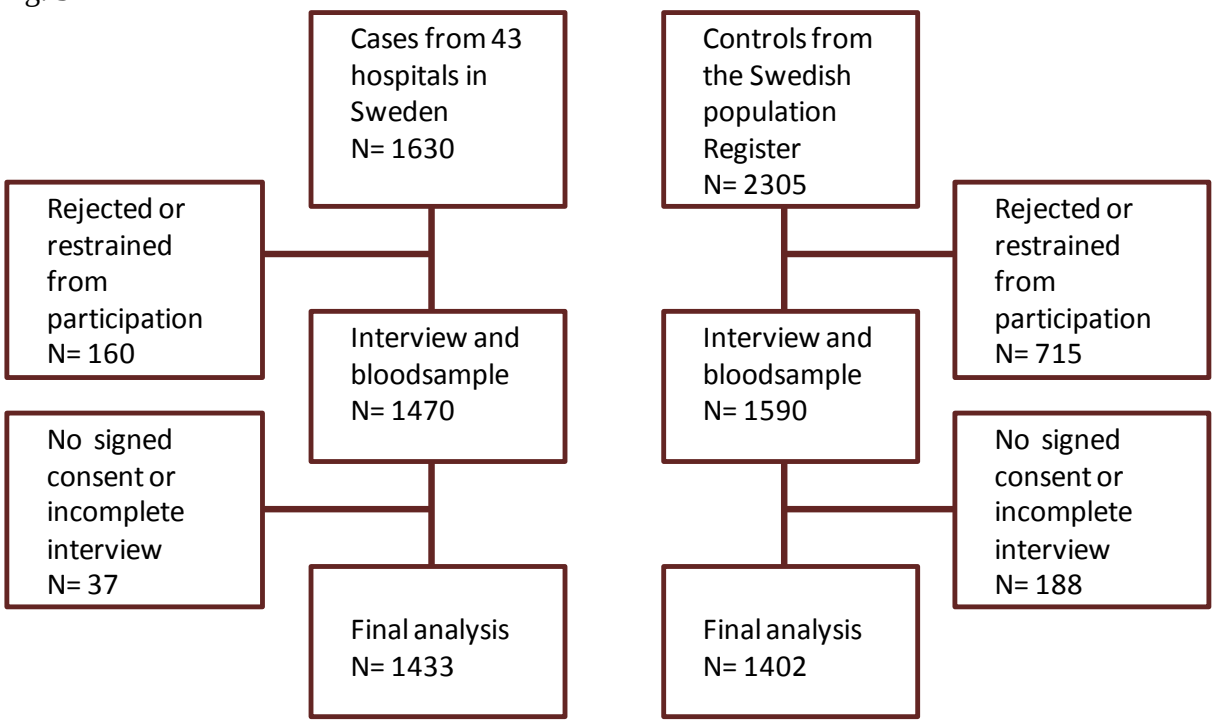
4.2.1 TEHS

In the TEHS-study, 1470 cases and 1590 controls were prospectively recruited in Sweden from 2003 to 2009. All cases were women between 18 and 64 years with a symptomatic first episode of deep vein thrombosis (DVT) of the lower limb or pelvis, or pulmonary embolism (PE). Female controls were continuously and randomly selected from the Swedish population register and frequency-matched to cases according to age. The date of diagnosis for women with VTE (cases), and the day of interview for controls, was set as the index date. Women with previous VTE, active cancer within the past five years or pregnancy within the past three months were not eligible for the present study. Telephone interviews with the participants were performed by experienced nurses within three months of the diagnosis of VTE for cases and at the index date for controls to collect data on life style factors and environmental risk factors for VTE. Genetic risk factors were identified by SNP-analyses on blood samples taken at the time of inclusion in the study.

In women eligible for the TEHS-study, the diagnosis of VTE had to be based on objective radiological methods. For women with a DVT the diagnostic methods used were venography or colour Doppler ultrasonography. Pulmonary embolism was verified by CT-scan of the thorax, pulmonary angiography or by perfusion- ventilation scintigraphy. To be classified as a symptomatic VTE, anticoagulant treatment should have been initiated. A pre-study inventory was performed by contacting all forty-three hospitals asking for their management of VTE. Thirty-two hospitals had a centralised management of VTE at departments of Internal Medicine or Haematology and eligible women were identified by a study coordinator at the department. At the eleven hospitals without centralised management of patients with VTE, potential study participants were identified through registers of referrals kept at each Department of Radiology and Department of Clinical Physiology. A copy of the radiology report was sent to the coordinating Centre where the research nurses contacted the clinician responsible for the potential case to make sure that it was ok to contact the woman. All women eligible for inclusion received both written and oral information about the study.

Controls were randomly selected from the Swedish population register and were contacted and enrolled according to the same procedure as previously described for cases, figure 3.

Fig. 3



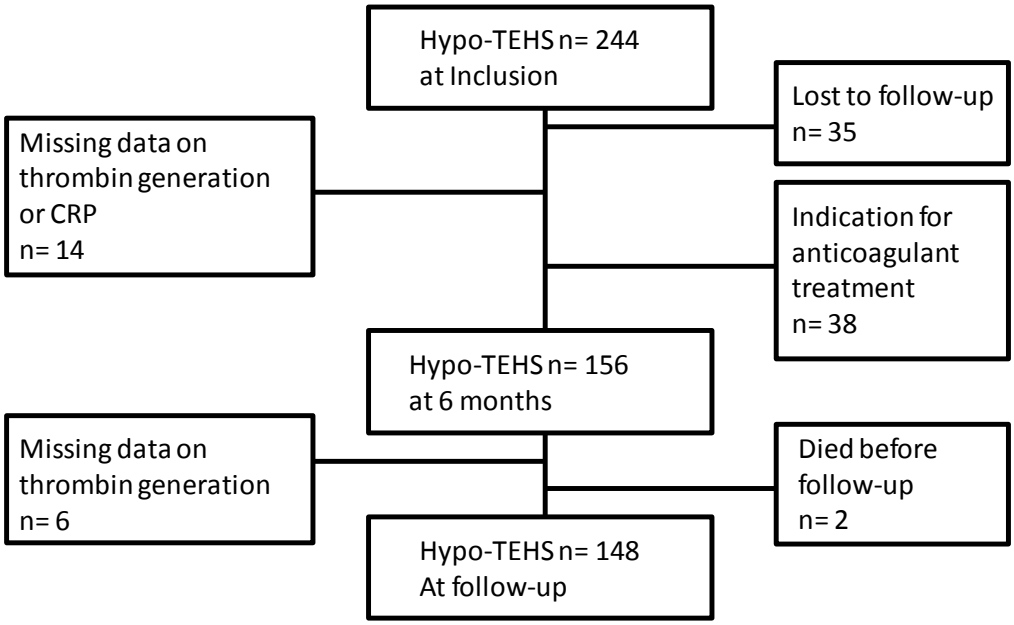
4.2.2 hypo-TEHS

243 consecutive female patients included as cases in TEHS were also included in the sub-study hypo-TEHS at the time of VTE diagnosis. The inclusion and exclusion criteria for hypo-TEHS were the same as for TEHS. The patients were recruited at four hospitals in Sweden (Södersjukhuset in Stockholm, Karolinska Universitetssjukhuset Solna, Linköpings Universitetssjukhus in Linköping and Sahlgrenska Universitetssjukhuset in Gothenburg) from 2003 to 2009.

All patients were included by the treating doctor or a nurse at the department of haematology. The study participants were followed up at six months after the diagnosis of VTE and left blood-samples at least three weeks after cessation of anticoagulant treatment. Another follow up concerning recurrence was conducted 24 months after inclusion and finally at the time of closing the study in 2009. All recurrent events were objectively verified by the same diagnostic work-up as at inclusion. Medical records were reviewed to confirm the diagnosis of symptomatic recurrent VTE that had indication for resumed anticoagulant treatment. Patients with clinical indication for long-term treatment, such as protein S, protein C or antithrombin deficiency, positive lupus anticoagulant or severe idiopathic PE were not included in this study (n=38). This was due to ethical concerns about withholding treatment to get plasma samples suitable for the analyses of thrombin generation. None of the patients in hypo-TEHS received estrogen-containing treatment during the follow-up period or at the time of blood-sampling.

Out of the initially identified 243 patients, 35 patients were lost to follow up at 6 months. Thirty-eight patients had clinical indication for long term treatment with anticoagulants. We were unable to perform measurement of thrombin generation in 11 patients and another three patients had missing samples for evaluation of CRP. In this way a total of 156 patients with complete follow up and measurements of thrombin generation were eligible for analysis in paper II. During the time of follow up of the hypo-TEHS patients, two patients died and six patients had missing data or insufficient amount of plasma for measurements of thrombin generation. One hundred and forty eight patients were left to analyse concerning the data on recurrence presented in paper III, figure 4.

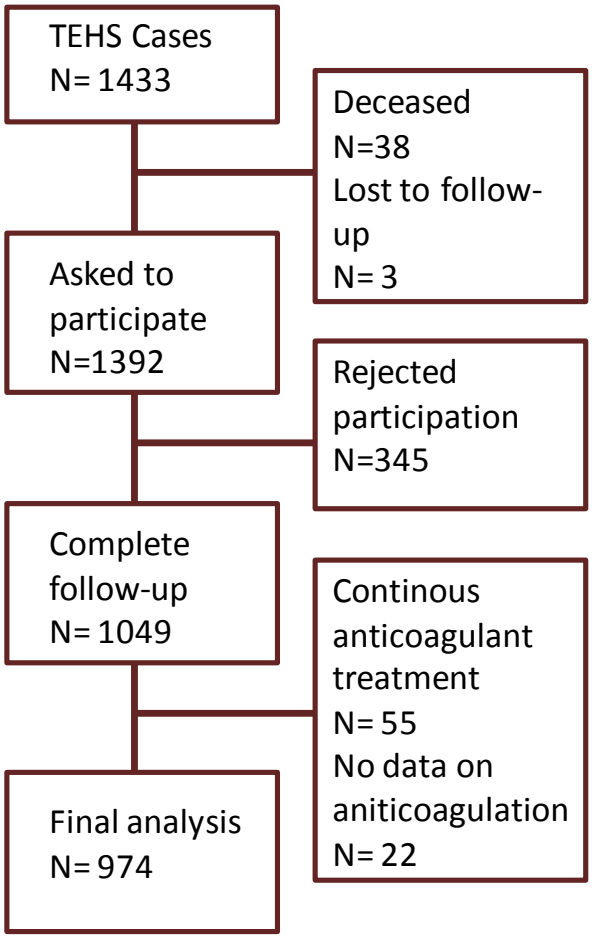
Fig 4.



4.2.3 TEHS follow-up

A follow up of all cases included in TEHS was conducted in 2011. Women on continuous anticoagulant treatment were not eligible for the recurrence-analysis of the follow-up study since they were not considered to be at risk of recurrence. Out of the 1433 initially included women, 38 had died since their inclusion in TEHS. The cause of death was reviewed and it was not related to VTE in any of the cases. Thirteen women died from cancer, 11 from cardiovascular disease and the remaining 14 from other causes. Three patients had emigrated from Sweden leaving 1394 living patients who were asked to participate in the follow-up study. Together with the request to participate a questionnaire was sent by mail to all 1394 women. A total of 345 patients rejected participation, 53 were on continuous anticoagulant treatment and 22 patients did not answer the question about current anticoagulant treatment, leaving 974 women at risk of recurrence to follow-up, figure 5.

Fig. 5



4.3 DATA COLLECTION

4.3.1 TEHS

4.3.1.1 Exposures

Data on exposures of cases and controls was collected through a telephone interview using a structured questionnaire. Data on acquired risk factors such as surgery, plaster/ cast treatment and use of CHC or HT was assessed for the three months preceding the index date. To support the memory, a catalogue with pictures of all oral contraceptives and hormones available in Sweden since the 1960 were sent to the women before the interview. Data on family history of VTE was collected for all participants with information on previous VTE in mother, father and siblings of the study participant. Data on height and weight was collected for the time of the interview.

4.3.1.2 Blood sampling

The women who accepted participation in the TEHS-study were instructed to donate a blood sample of 7.5 ml at the nearest health care provider. For the blood sample a "blood sample kit" including bar code and test tubes was sent to the women for them to bring to the health care provider. The samples were then sent by overnight mail to the laboratory at the Medical Products Agency in Uppsala, Sweden.

4.3.2 Hypo-TEHS

4.3.2.1 Exposures

In addition to the data collected in TEHS all participants in hypo-TEHS filled in a short inclusion-form consisting of questions regarding family history of VTE, known thrombophilias, current hormonal treatment and durations of symptoms of VTE at inclusion. The participants were asked about current medication including anticoagulants and hormonal treatment at each follow up occasion. Data on recurrent VTE was also assessed at each follow up.

4.3.2.2 Blood sampling

Six months after inclusion, at least three weeks after cessation of anticoagulant treatment, blood samples were donated by all hypo-TEHS participants. Samples were collected into vacutainer tubes containing 3.2 % sodium citrate and centrifuged twice at 2000xg for 15 minutes at 15°C. The platelet poor plasma was snap-frozen and stored at -70ºC until testing.

4.3.3 TEHS follow-up

The patients who participated in the follow up study of TEHS answered a questionnaire containing questions on current medication, duration of previous or current anticoagulant treatment, hormone therapy with CHC or HT and recurrent events of VTE. Information on recurrent VTE was obtained both from the questionnaire and from data recorded in the Swedish Patient Register, a nationwide registry held by the National Board of Health and Welfare in Sweden. All recurrent events were identified by questionnaire, register or both. After identification, all possible recurrent events were objectively verified by review of the medical records. To be considered as a recurrent event an objective confirmation of the diagnosis had to be done with any of the same radiological methods as at inclusion and the event had to be regarded as having indication for resumed anticoagulant treatment. The follow-up period started at the time of the discontinuation of anticoagulant treatment after the first event of VTE and patients were censored either at first recurrent thrombotic event or at the date of answering the questionnaire. If information on the duration of anticoagulant treatment after the first event of VTE was missing, women with an index VTE as DVT were assumed to be treated with anticoagulants for 6 months and women with index PE for 12 months according to the clinical guidelines during the time of inclusion in Sweden.

4.4 LABORATORY METHODS

4.4.1 Genetic analysis

Genetic analysis was performed to identify women with the single nucleotide polymorphisms factor V Leiden (G1691A; Arg506Gln) and prothrombin gene mutation (G20210A). DNA was prepared from 5 ml of blood using QIAGEN FlexiGene DNA kit. The genotyping was performed at the Royal Institute of Technology in Stockholm. Initially target genes were amplified by PCR-technique and the genotyping was made by means of Pyrosequencing (7).

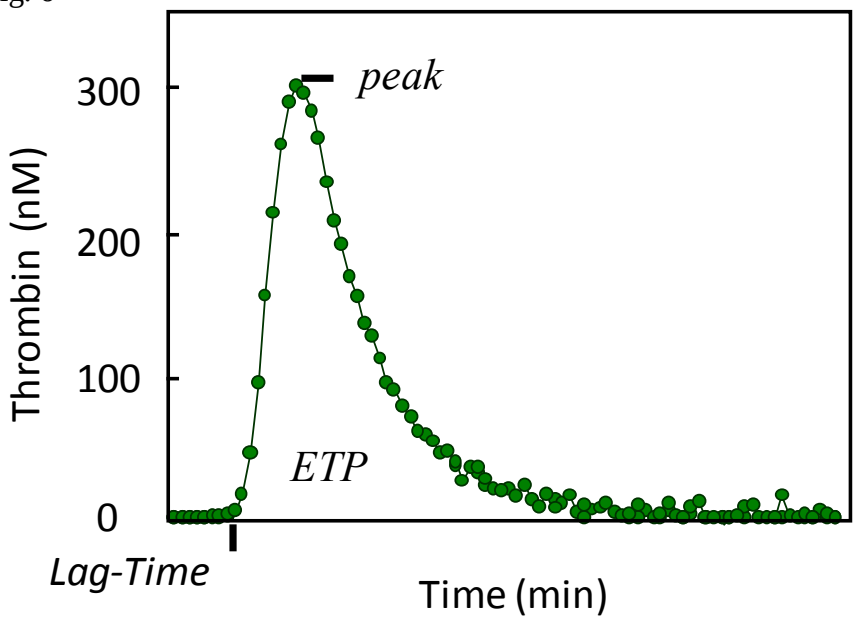
4.4.2 Thrombin generation

Thrombin is the end stage of the coagulation cascade and the capacity to generate thrombin reflects the interaction of the different coagulation proteins and the natural anticoagulant systems. In this way a prothrombotic or haemophilic state of the coagulation system can be captured in one test. Different types of assays are available (162). The fluorogenic Calibrated Automated Thrombogram (CAT) was used in paper II and III (169). The chromomeric Innovance ETP-assay was used in paper II.

4.4.2.1 The thrombin generation curve

The outcome variables in measurement of thrombin generation are related to the thrombin generation curve, figure 6.

Fig. 6



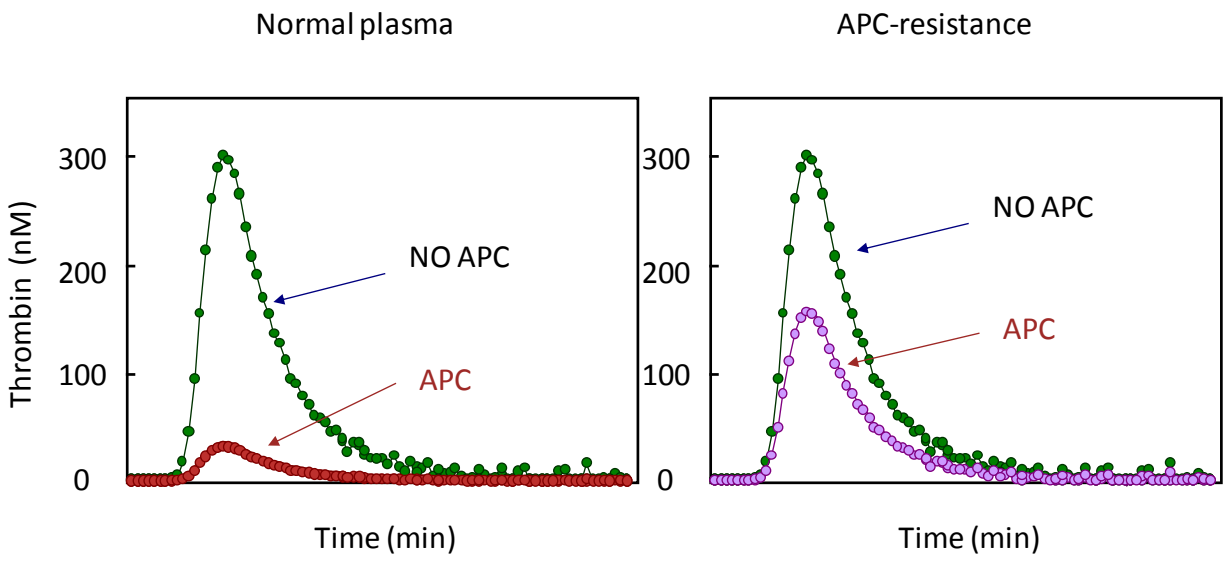
The most important parameters are:

- **Lag time** - the time after initiation of the coagulation cascade but before the burst of thrombin. This parameter correlates with the clotting time.
- **Peak height**- the maximum level of thrombin that is measured.
- **Endogenous Thrombin Potential (ETP) -** the area under the curve which represents the total amount of enzymatic work of active thrombin.

4.4.2.2 APC-resistance

Resistance to activated protein C (APC) makes the coagulation system less sensitive to the natural anticoagulant activity of APC. The most well known reason for APC-resistance is the factor V Leiden mutation as previously described (101). APC-resistance can also be present without the factor V Leiden polymorphism and in patients using CHC or HT (17, 170). The latter phenomenon is referred to as acquired APC-resistance. To evaluate APC-resistance an aPTT-based test was developed by Dahlbäck et al in 1993 (171) . In this test the aPTT is determined in the absence and in the presence of APC and the APC-resistance is expressed as the ratio between the two measurements on the same plasma sample. Only a small amount of thrombin is needed to reach clotting in plasma, which is the endpoint in the aPTT-test. This means that it is mainly the initiation phase of coagulation that is evaluated with the aPTT-based test. Thrombin generation-based tests of APC-resistance have also been developed in later years (170, 172). As the endpoint of the thrombin generation test measured in ETP takes in to account all generated thrombin it evaluates the initiation, propagation and termination of coagulation. This makes it more sensitive to the natural anticoagulant systems like the APC-system than the aPTT-based test. The thrombin generation based APC-resistance test using CAT is based on measurements of ETP in the presence ($ETP_{+\ APC}$) and in the absence ($ETP_{-\ APC}$) of APC in plasma samples from the same individual, figure 7. The amount of added APC is chosen to reduce the ETP by 90 % in normal plasma. The result is then expressed as a sensitivity ratio (APCsr) between the measurements. A patient with an APC-resistant phenotype will have less inhibition of thrombin generation after APC is added and will therefore yield a higher value of APCsr. The thrombin generation based APC-resistance test was used in paper III.

Fig. 7



4.5 STATISTICAL METHODS

Binary logistic regression analysis was used to analyze the case-control data of paper I. Odds ratios (OR) for VTE and their corresponding 95% confidence intervals (CI) were calculated in univariate and multivariable models. A value of $p < 0.05$ was considered as a statistically significant association between the studied exposures and the outcome VTE.

In paper II, one way analysis of variance (ANOVA) was used to compare parameters related to inflammation and thrombin generation in obese and non obese patients. Spearman's correlation test was used to estimate correlation between coagulation factors/inflammatory markers and thrombin generation. To investigate the determinants of the increased thrombin generation in obese women we performed univariate and multivariable linear regression analysis. The assumptions of normality, equality of variance (homoscedasticity) and linearity were investigated for the multivariable models as was potential outliers and multicollinearity. A two sided probability value of $p < 0.05$ was considered statistically significant.

In paper III and IV cumulative incidence of recurrent VTE was estimated using Kaplan-Meier method with censoring at time of recurrent event or end of follow-up. Groups were compared using log-rank test. A Cox proportional hazard model was used to calculate both crude and multivariate adjusted hazard ratios (HR) for recurrence with their corresponding 95% confidence intervals. All statistical analyses were performed by using the SPSS for Windows, version 17-20 (SPSS Inc., Chicago, Ill).

5 RESULTS

5.1 PAPER I

The study presented in paper I was performed on 1288 women and 1327 age- matched controls included in TEHS with complete data on family history of VTE and genetic risk factors. In total, 345 cases and 189 controls had a positive family history of VTE. A positive family history was associated with an increased risk of VTE with an adjusted odds ratio (OR) of 2.2 (95% CI 1.7-2.7). Among the women with a positive family history 147 (28%) were carriers of the Factor V Leiden or the prothrombin G20210A mutation. The sensitivity for detecting any of these thrombophilias with a positive family history was 29% and the corresponding specificity was 82%. Patients who were both carriers of any of the mentioned thrombophilias and had a positive family history had a more pronounced risk of VTE than women with only one of these risk factors.

Women who were users of either CHC or HT, and who had a negative family history, had a significantly higher risk for VTE than non users with a negative family history (OR 8.3 and 2.6 respectively). In women with a positive family history who were users of either CHC or HT the OR was further increased with OR 15.3 and 5.9 respectively. In women who were subject to a surgical procedure or plaster cast treatment but had a negative family history of VTE, the risk of VTE was increased 16-fold compared to women without surgery / cast with negative family history. A positive family history in combination with surgery or cast treatment was associated with substantially higher risk of VTE with OR 66.5 (95% CI 21-213). The impact of positive family history on risk of VTE and its role in HT-, CHC- and surgery/plaster related risk is shown in table 1.

Table 1

	Controls n=1288 (%)	Cases n= 1327 (%)	OR * (95% CI)	OR_a (95% CI)
Family History				
Negative family history	1099 (85)	982 (74)	Reference	Reference
Positive family history	189 (15)	345 (26)	2.2 (1.8-2.7)	2.2 (1.7-2.7)†
Family history and Hormones				
Negative family history No HT or CHC	911 (71)	582 (44)	Reference	Reference
Positive family history No HT or CHC	165 (13)	250 (19)	2.3 (1.6-2.9)	2.2 (1.7-2.8)‡
Negative family history Treatment with HT	95 (7)	132 (10)	2.0 (1.5-2.7)	2.6 (1.9-3.6)‡
Positive family history Treatment with HT	18 (1.4)	55 (4.1)	4.5 (2.6-7.7)	5.9 (3.3-11)‡
Negative family history Treatment with CHC	93 (7)	268 (20)	5.3 (4.0-7.2)	8.3 (5.9-12)‡
Positive family history Treatment with CHC	6 (0.5)	40 (3.0)	11.7 (4.9-28)	15.3 (6.1-38)‡
Family history and Surgery/ cast				
Negative family history No surgery/ plaster	1078 (84)	665 (50)	Reference	Reference
Positive family history No surgery/ plaster	163 (13)	211 (16)	2.3 (1.8-2.9)	2.1 (1.7-2.7)§
Negative family history Surgery/ plaster	44 (3)	362 (27)	13.2 (9.5- 18)	16.4 (11.6-23)§
Positive family history Surgery/ plaster	3 (0.2)	89 (7)	62.4 (20-199)	66.5 (21-213)§

*Adjusted for age †Adjusted for surgery/ plaster, CHC or HT, G20210A and/ or Factor V Leiden, age and BMI. ‡Adjusted for surgery/ plaster, G20210A and/ or Factor V Leiden, age and BMI. §Adjusted for factor V Leiden and/ or GA20210A, age, BMI and use of CHC or HT. Family history in models with hormones and surgery/ plaster as a dichotomous variable (yes/no)

5.2 PAPER II

The study population of interest in paper II consisted of 118 non obese women and 38 obese women, with a first event of VTE included in the hypo-TEHS study. Blood samples were collected at least six months after the diagnosis of VTE and at least three weeks after cessation of anticoagulant treatment. The clinical characteristics and mean values in coagulation factor levels and levels of inflammatory parameters of the patients are shown in table 2.

Table 2

Clinical Characteristics		BMI <30 n= 118	BMI ≥30 n= 38	p-value
Age	mean years (range)	45 (18-64)	47 (18-64)	0.20
Family history of VTE	n (%)	36 (31)	9 (24)	0.47
PE	n (%)	29 (25)	11 (29)	0.59
Prox DVT	n (%)	12 (10)	7 (18)	0.18
Dist DVT	n (%)	77 (65)	20 (53)	0.16
Smoking	n (%)	35 (30)	8 (22)	0.34
Hormones at diagnosis	n (%)	46 (40)	10 (26)	0.16
Trauma/surgery	n (%)	46 (40)	14 (37)	0.81
Factor V Leiden	n (%)	21 (18)	7 (20)	0.83
GA20210A mutation	n (%)	14 (12)	5 (14)	0.81
Coagulation factors/ inflammatory parameters:				
Prothrombin (IE/ml)	mean (SD)	1.08 (0.17)	1.17 (0.13)	0.008*
Factor VII (IE/ml)	mean (SD)	1.08 (0.39)	1.20 (0.32)	0.11
Factor VIII (IE/ml)	mean (SD)	1.47 (0.47)	1.64 (0.53)	0.06
CRP (mg/l)	mean (SD)	2.32 (5.7)	4.79 (5.0)	0.02*
PAI-I (IE/ml)	mean (SD)	20.32 (11.1)	31.50 (17.9)	0.000*
Fibrinogen (g/l)	mean (SD)	3.58 (0.70)	4.26 (0.71)	0.000*
d-dimer (mg/l)	mean (SD)	0.23 (0.39)	0.24 (0.35)	0.86

Differences in continuous variables explored with one-way ANOVA, dichotomous variables with Chi square test, SD: standard deviation, PE: Pulmonary Embolism, Prox: proximal DVT, Dist: distal DVT (under knee). All risk factors assessed at the time of thrombosis. * indicate significant differences on the $p < 0.05$ level.

The parameters of thrombin generation in CAT varied between obese and non obese patients with a prolonged lag-time and higher ETP- values in the obese women, when 10 pM of TF was used to trigger thrombin generation. When BMI was analyzed as a continuous variable, a positive association was observed between Lag-time, ETP and BMI at both high (10 pM) and low (1 pM) TF, table 3. When the chromogenic "Innovance® ETP-assay" was used to measure thrombin generation, the same relation

between BMI and C-max (corresponding to peak height in CAT) was observed. Moreover, the obese women had higher ETP and C-max than non obese also in this method although no difference in lag-time was detectable.

Table 3.

Method	Parameter		BMI <30 (n=118)	BMI ≥30 (n=38)	P-value
CAT 10 pM TF	Lag-time	(min)	1.86	2.04	0.005*
	ETP	(nM*min-1)	840	934	0.001*
	Peak	(nM)	279	287	0.50
CAT 1 pM TF	Lag-time	(min)	5.3	6.2	0.003*
	ETP	(nM*min-1)	540	596	0.13
	Peak	(nM)	74	80	0.47
Innovance®	Lag-time	(sec)	25	25	0.79
ETP-assay	ETP	(mA)	442	483	0.000*
	C-max	(mA/min)	117	127	0.002*

Significance of differences between the groups is tested with one-way ANOVA with p-values in the right column. TF: tissue factor, ETP: endogenous thrombin potential, CAT: calibrated automated thrombography. *indicate significant differences on the p <0.05 level.

The determinants of thrombin generation in our study population were investigated by stepwise linear regression. We calculated β-values in crude and adjusted models for the relation between obesity and each of the thrombin generation parameters that were significantly influenced by obesity in the previous analysis. Factors that had substantial impact on the standardized β-value of the model were considered to be the most important determinants of the parameter of interest. Of the factors analyzed in this study, fibrinogen and prothrombin were the most important determinants of the association between obesity and increased thrombin generation. The main determinant for the prolonged lag-time in measurements with CAT was increased fibrinogen and the main determinant of increased ETP was prothrombin. In the Innovance ETP-assay the determinant of the C-max was correlated with both fibrinogen and prothrombin. CRP was also significantly associated with all thrombin generation parameters but this association was independent of obesity status.

5.3 PAPER III

In paper III, 148 patients from the hypo-TEHS study were followed for a mean time of 46 months after cessation of anticoagulant treatment of a first event of VTE. Thirteen patients suffered from recurrence which corresponds to 8.7% of the study population. We wanted to evaluate if APC-resistance in the absence of factor V Leiden was correlated with recurrent VTE. Data on the patients without factor V Leiden (n=117 showed that peak height$_{+ APC}$, ETP$_{+ APC}$ and APCsr were significantly associated with increased HR for recurrence, table 4. Furthermore we analyzed thrombin generation triggered with a low concentration of TF (1pM) in the presence and in the absence of anti-Protein S and TFPI-antibodies. In this analysis we found that thrombin generation in the presence and in the absence of protein-S antibody was correlated with the risk of recurrence. The measurements in the presence of TFPI-antibody did not correlate with recurrence, table 4.

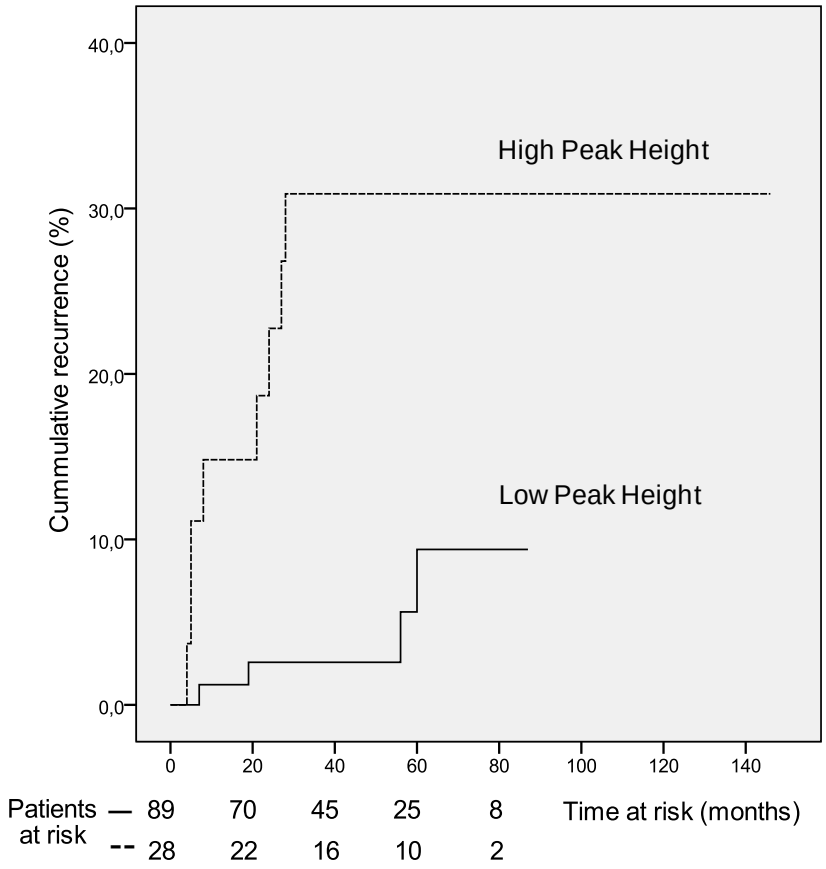
Table 4

		HR	95 % CI	P-value
TF 10 pM	Peak height –APC	1.07	0.95-1.21	0.26
	ETP –APC	1.00	0.95-1.03	0.53
	Peak height +APC	1.21	1.10-1.33	<0.001 *
	ETP+APC	1.06	1.02-1.10	<0.001 *
	APCsr	1.76	1.29-2.42	<0.001 *
TF 1 pM	Peak height	1.16	1.01-1.33	0.029 *
	Peak height +anti protein-S	1.42	1.12-1.80	0.003 *
	Peak height +anti TFPI	1.15	0.91-1.46	0.24

Multivariate Cox proportional Hazards model with adjustment for age, BMI> 30, localization of index event and the GA20210A mutation. TG-parameters analyzed as continuous variables with HR for increase of 10 nM in the peak height, 10nm*min in the endogenous thrombin potential (ETP) and 1.0 in the APC sensitivity ratio (APCsr). Tissue Facctor (TF), * $p < 0.05$

Furthermore, we wanted to assess if we could identify a group of high risk women by the level of APC-resistance. We found that patients with the highest quartile of peak height$_{+ APC}$ with HR 6.3 (95% CI 1.9-20.8). ETP$_{+ APC}$ had an increased risk of recurrence with and 4.4 (95% CI 1.3-13.1) and as expected the APCsr also correlated with increased risk of recurrence with HR 4.9 (95% CI 1.5-15.8) as shown in figure 8.

Fig. 8



5.4 PAPER IV

In paper IV, 974 of the women included as cases in the original case-control study TEHS were followed-up for a median time of 5.2 years after the first event of VTE. During follow-up 102 women had an objectively confirmed recurrent VTE corresponding to an overall annual rate of recurrence of 2.0%. All patients had the highest risk of recurrence during the first year. Cumulative incidence of recurrence is shown in table 5.

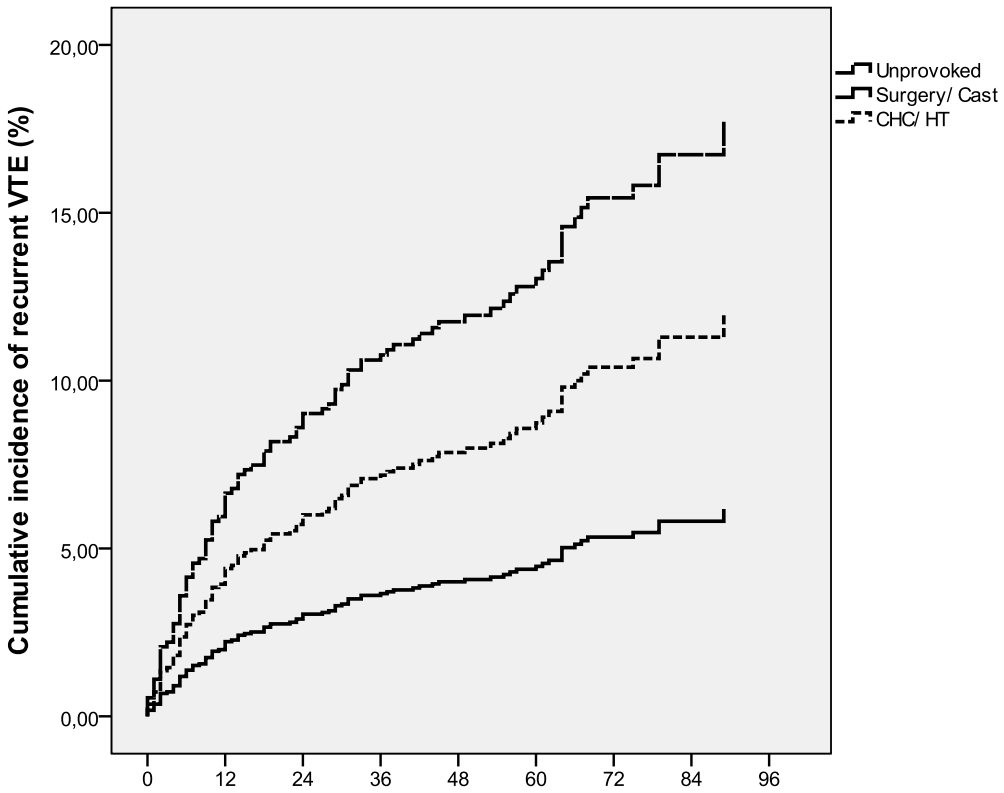
Tab. 5

	All women n=974	Women with Surgery/ Cast n= 350	Women with CHC/HT n =272	Women with Unprovoked VTE n= 352
1 year	4%	2%	5%	7%
2 years	6%	3%	7%	10%
3 years	8%	3%	7%	13%
4 years	9%	3%	8%	15%
5 years	9%	4%	9%	15%
6 years	11%	6%	10%	18%

The risk of recurrence was higher in obese than in non-obese women with HR 1.8 (95% CI 1.2-2.7) for obese women. This association was more pronounced in women under the age of 50 years where obesity was associated with increased risk of recurrent VTE with crude HR 2.4 (95 % CI 1.3-4.3) and HR_a 2.5 (95% CI 1.3-4.7) compared to non-obese. Carrier ship for factor V Leiden and / or the prothrombin gene mutation G20210A was not significantly associated with recurrence HR_a 1.3 (95% CI 0.9-2.0). Neither was a positive family history, HR_a 1.2 (95% CI 0.8-1.8) or the age of the patient.

When patients with unprovoked, surgery/ cast-provoked and hormone-provoked VTE were compared, patients with unprovoked first VTE had the highest risk of recurrent events followed by patients with hormone associated first VTE and patients with surgery/ cast-provoked VTE, figure 9.

Fig.9



Patients at risk:							
Surgery/ cast	350	343	329	289	228	166	61
CHC/HT	272	258	244	216	178	141	68
Unprovoked	352	328	304	254	197	146	61

6 DISCUSSION

6.1 SELF-REPORTED FAMILY HISTORY AND RISK OF VTE

VTE is one of the leading causes of death in younger women in developed countries. Although the absolute risk is low many of the incident cases could, at least in theory, be avoided since they are related to transient risk factors. To be able to reduce the incidence of VTE in general, and fatal PE in particular, better ways to identify women at increased risk for VTE is crucial. Several of the transient risk factors related to VTE in women provide beneficial effects that outweigh the risk of VTE in most cases. The challenge is to find easy and cost effective ways to identify the individuals who are at high risk of developing VTE in connection to the specific transient risk factors.

Many studies have set out to find ways to predict VTE related hormonal treatment like CHC and HT (41, 43, 108). Most reports have found that genetic thrombophilias are related to multiplicative risk of VTE in women with hormonal treatment. Screening with thrombophilia-testing prior to prescription of CHC or HT is however not considered cost effective (65). Several studies have evaluated the use of a positive family history as a proxy for genetic thrombophilias (88-90). In general, most studies reported a low sensitivity to find genetic thrombophilia by family history (88, 89, 103). During the past years reports on family history as an independent predictor of VTE in patients with both genetic and acquired risk factors have been published (91, 173). In paper I of this thesis we were able to specifically evaluate the role of a positive family history on the risk of VTE in women with hormonal- or surgery/ plaster treatment. Our results showed that a positive family was correlated with increased risk of VTE in women in these specific risk situations. The increased risk was persistent both in multivariable models adjusting for thrombophilia-status as well as in stratified analysis on women with and without thrombophilias. The results of the TEHS-study implicate that the risk associated with family history is at least in part independent from the risk related to known thrombophilias.

Surgery and plaster cast treatment are known and well established risk factors for VTE in women (66). Recently published data from the “One million women study” in the UK comprising 947000 women showed that the risk of VTE was increasing up to 12 weeks after surgery with 1/140 middle aged women suffering from VTE after a surgical procedure (174). The highest risk estimates were correlated with orthopedic surgery and the study highlights the importance of surgery as a strong predictor of VTE in middle aged women and the room for improvement in prevention. Previous studies have shown that thrombophilias seems to be associated with a moderate increased risk of VTE in connection with surgery/ plaster but testing prior to surgical procedures is not a feasible option (36, 175-177). In paper I we evaluated the role of a positive family history of VTE in estimating the risk of surgery/ plaster related VTE. We found that the risk of VTE was more than three-fold in women with a positive family history and

conclude that is might be used to identify high risk patients who could benefit from extended anticoagulant prophylaxis.

6.2 OBESITY AND VENOUS THROMBOEMBOLISM IN WOMEN

Another strong risk factor of VTE in both men and women is obesity. Some data implicate that obesity is an even stronger risk factor for VTE than for coronary heart disease and stroke (80, 82, 178). The mechanisms of how obesity increases the risk of VTE are not fully understood. Some studies report increased venous pressure in obese patients while others find that obesity increases some coagulation factors inducing a hypercoagulable state (84, 141, 179, 180). In the Hoorn study, a study of thrombin generation in relation to body composition, obesity was correlated with increased thrombin generation particularly in women (181). Previous publications from the TEHS-study showed that obesity is a risk factor for VTE in our study population (182). Data from THES-follow up (paper IV) confirmed that obesity is also a predictor of recurrent VTE among women, particularly in women below 50 years of age.
To explore the impact of obesity on the coagulation system of women with VTE we measured coagulation factor levels, inflammatory parameters and thrombin generation in patients from the sub study hypo-TEHS as presented in paper II. Obese women had significantly higher levels of thrombin generation than non obese with consistent results in two different methods of measuring thrombin generation. The endogenous thrombin generation potential (ETP) was the parameter that showed the most equivalent results. Obese subjects also had increased levels of coagulation factors related to inflammation such as prothrombin, fibrinogen and PAI-I. Previous publications of data from a healthy population has shown that several of these proteins are the main determinants of thrombin generation (183). In our study on women with VTE, increased levels of prothrombin was the main determinant of the increased ETP on obese subjects. Increased levels of fibrinogen were correlated with increased lag-time in obese patients when CAT was used to measure thrombin generation in obese patients. The detected hypercoagulable state in obese women with VTE could represent a link between obesity, inflammation and increased risk of recurrent VTE.

6.3 THROMBIN GENERATION AND THE RISK OF RECURRENT VTE

Increased thrombin generation has been reported to correlate with increased risk of both first and recurrent VTE (156, 157, 159). In previous studies, thrombin generation has been measured under various conditions, with different amounts of tissue factor (TF) to trigger the onset of coagulation. Thrombin generation can also be measured in the presence and the absence of other important factors of the coagulation system, such as thrombomodulin, activated protein C and antibodies towards protein S and TFPI. In a study by Tripodi et al, elevated thrombin generation measured in the presence of Thrombomodulin was found to better predict recurrent VTE than measurements in the absence of thrombomodulin (159). Furthermore, thrombin generation measured in the

presence of Protac, a snake venom derived activator of protein C, correlated with increased risk of recurrence in an Austrian study (184). These findings indicate that thrombin generation based tests evaluating the function of the activated protein C (APC) system have the strongest correlation to recurrent VTE. Resistance to APC is known to result from genetic causes like the factor V Leiden mutation and also from acquired APC-resistance in women who are users of CHC or HT. In paper III we showed that a thrombin generation based test of resistance to APC was correlated with increased risk of recurrence of VTE in women also in the absence of factor V Leiden and hormonal treatment. Moreover, we found that thrombin generation triggered with low concentrations of TF was also significantly correlated with the risk of recurrence. Thrombin generation triggered with low TF is particularly sensitive to changes in the Protein S and TFPI regulated part of the anticoagulant system. Under these conditions measurements of thrombin generation in the presence of anti protein S antibodies correlated with recurrence but measurements in the presence of TFPI-antibodies did not. Protein S is a promoter of the down regulation of the TF-derived thrombin generation regulated by TFPI. Hence, changes in the TFPI seems to be of importance in the malfunction of natural anticoagulant systems connected to increased risk of recurrent VTE in our study population.

6.4 RECURRENT VTE IN YOUNG AND MIDDLE AGED WOMEN

The clinical challenge after a first event of VTE is to recognize the individuals who are at high risk of recurrence and would thereby benefit from long term anticoagulant therapy.

To further evaluate the overall risk of recurrent VTE, and to estimate the risk related to a first hormone associated VTE in women, we conducted a long term follow up of all patients included as cases in the initial case-control study TEHS. The results are presented in paper IV and show that the overall risk of recurrence in women 18-64 years is low with an annual incidence of 2%. This is lower than the estimated risk of bleeding complications related to anticoagulant therapy and raises questions about if women in this age group really benefit from secondary prevention with anticoagulants. The incidence in the first year after cessation of primary anticoagulant therapy was however higher especially in idiopathic cases, indicating that there is a high risk group that needs to be recognized. In recent years, several studies have confirmed that the risk of recurrent VTE is higher in men than in women (134). An explanation of this difference has been a low risk of recurrence in first VTE related to female hormones in women. This is however controversial and the sex difference between men and women remains in studies where hormone-associated first event have been excluded (135, 136). Some studies have found that the risk is reduced after a first VTE provoked by HT or CHC as compared to unprovoked VTE but in several studies there was no significant difference in risk of recurrence between women with a first CHC/ HT related VTE and a first unprovoked event (81). Available studies have reported conflicting results on if a first VTE should be considered to be provoked or not. In 2003

Baglin et al showed that patients (both men and women) with a non-surgical trigger factor had significantly lower risk of recurrence than patients with unprovoked VTE (126). The group of non-surgical triggers included use of CHC, HT and long haul travel but also plaster cast and fractures which make it hard to draw any conclusions regarding the risk recurrence in hormone associated VTE. In a large Austrian cohort study on recurrent VTE, 175 women with first VTE during use of CHC were analyzed. The risk of recurrent VTE was not lower in these women compared to women with unprovoked VTE in the same age group (135). Data from the PREVENT-study reported a 46% reduction in risk of recurrence in 129 women with hormone associated first VTE compared to 109 women with unprovoked VTE after 2.1 years (138). These findings were however not statistically significant and the authors argued that it might be due to short follow up period. In a patient-level meta analysis 2008, Douketis et al reported a risk reduction concerning recurrence in VTE of 50% in a pooled number of 340 women with hormonal treatment compared to 801 women without hormonal treatment at first VTE (HR 0.5, 95% CI 0.3-0.8) (134). However, the Cox proportional Hazards model did not include any multivariate adjustments to control for potential confounding by age, BMI or thrombophilia. Moreover, Douketis et al showed that use of CHC or HT at first event is useful in predicting recurrence together with other clinical and laboratory characteristics in a prediction score (185). In a study of young women less than 45 years of age in Austria, 361 women were evaluated for recurrent VTE. The rate of CHC use at first VTE was 52% and a total of 141 patients had a recurrent even within 11 years. Use of CHC at first VTE was not significantly associated with any reduced risk for recurrence in this population; neither were other triggering factors like surgery or plaster cast (186). Data from the REVERSE-study on 314 women of showed no significant difference in risk if recurrent VTE between users of CHC or HT at first VTE compared to non users (137). The authors concluded that hormone associated VTE cannot be regarded as provoked VTE and further risk assessment is needed in this group. In paper IV of this thesis we have available data on 970 women less than 65 years of age with a total follow up period of 4917 patient-years. Our data suggest that the risk of recurrence in hormone related VTE is not the same as either idiopathic or surgery-related VTE, but somewhere in between.

6.5 METHODOLOGICAL CONSIDERATIONS

The TEHS study is one of the largest clinical studies performed on a female population including life style factors and genetic information. The strength of the study is the large sample size and the aim to study risk factors specific for this population of young and middle aged women. Some methodological considerations do however merit further discussion. The case control design was chosen since the outcome of interest, namely VTE, is relatively rare in this specific population. The internal validity is depending on the potential of systematic error and random error. Systematic error, or bias, can be caused by how the study objects are selected, the way the variables were

measured (information bias) and confounding factors. The population based design with 43 including sites all over Sweden means that the external validity is good if the internal validity is high.

6.5.1 Selection bias

Selection bias can never be ruled out in a case control study but minimized by a thorough study design. The participation rate was lower in controls than in cases which could represent a risk of selection bias towards women with any medical history who might be more prone to participate. Other possible selection criteria could be women with hormonal treatment who have read about the risk of VTE and would thereby be more prone to participate. The reported use of CHC and HT among the controls was however in the range as reported from the Swedish Prescribed Drug Register which argues against selection bias on hormonal exposure.
Selection bias could also be a problem in the TEHS-follow up where 345 women out of 1392 rejected participation in the follow up study. Patients with recurrence or other problems after their first VTE might be more interested in a follow up than patients who have no complications afterwards. Moreover, it is important to remember that patients with ongoing long term anticoagulant treatment since their first VTE were excluded from the analysis on recurrent VTE as they were not considered to be at risk which represents a selection bias. Selection bias in the sub study hypo-TEHS could arise if patients with more severe VTE were more interested in clinical follow up whereas those with fewer symptoms would prefer not to revisit the hospital for blood-sampling and follow-up visits.

6.5.2 Information bias

Misclassification and recall-bias are two forms of possible information bias of concern in a case-control study. In TEHS, a catalogue with pictures of the most common preparations of CHC and HT was sent to all cases and controls before the telephone interview to minimize recall bias and misclassification concerning hormone exposure. Furthermore all interviews were performed by trained research nurses who were using a structured questionnaire. In the laboratory part of hypo-TEHS the ambition was to conduct all measurements of the same variable at the same time with the same persons performing the measurements in the laboratory. The measurements of thrombin generation with consistent results using two different methods in two different laboratories suggest low risk of bias in that exposure variable. In the TEHS follow-up study, recall bias and misclassification concerning recurrent VTE was minimized by the use of information from both the nationwide Swedish Patient Register and the patient's statement of recurrence together with reviews of medical records.

6.5.3 Confounding

The word confounding refers to the confusion or mixing of effects from different exposures. A confounder needs to be associated with both the exposure and the outcome and it cannot be the result of the outcome. Potential confounders can be prevented in several ways in epidemiological studies. In the TEHS study, we used matching by age to avoid confounding by age in the analysis and we restricted the study to only include women 18-65 years. In the analysis of family history as a predictor of VTE in women with CHC/HT or surgery/plaster we controlled for known confounders in multivariable analysis. We confirmed the independent effect of family history by stratifying the analysis to patients by the presence of thrombophilia to estimate the risk in patients without factor V Leiden or prothrombin gene mutation. In the TEHS follow-up study we controlled for potential confounders by multivariable analysis and by performing stratified analysis on women below and over 50 years of age. The purpose of the latter was to be able to look at HT and CHC separately. In the analysis of hypo-TEHS, all risk estimates were adjusted for potential confounders in multivariable analysis.

6.6 SUMMARY AND FUTURE IMPLICATIONS OF THE RESULTS

In conclusion we investigated risk factors related to first and recurrent VTE in young and middle aged women. We found that assessment of family history can be useful in identifying women at increased risk of hormone, surgery and cast related VTE. If this is confirmed in future studies it could lead to better ways to prevent VTE in the mentioned risk situations. The overall risk of recurrence was low our study population of women, indicating that the gender of the patient should probably be integrated in the evaluation of duration of treatment. The highest risk of recurrence in young and middle- aged women was found in patients with idiopathic VTE, followed by hormone related VTE and last surgery/ cast related VTE which could allow for further improved risk stratification. Moreover, our data show that obesity is an important risk factor of recurrent VTE, especially in women under 50 years of age indicating that younger women might be the ones who would benefit from weight reduction counseling after a first VTE. Concerning the mechanisms behind the correlation between obesity and VTE we found that obesity was correlated with increased thrombin generation and inflammation suggesting an explanation to the increased risk of recurrent VTE. Thrombin generation was related to increased risk of recurrent VTE in this female population. Furthermore, APC-resistance in the absence of factor V Leiden was also related to increased risk of recurrence. Our results confirm that that thrombin generation is useful in estimating hypercoagulability related to venous thromboembolism.

7 CONCLUSIONS

- Self reported family history of VTE is correlated with increased risk of first VTE associated with hormones, surgery and cast treatment in women.

- Obesity in female VTE-patients is associated with increased thrombin generation and low grade inflammation.

- Obesity is a risk factor for recurrent VTE in women, especially in patients with an age below 50 years.

- APC-resistance in the absence of Factor V Leiden determined with CAT is related to increased risk of recurrent VTE.

- Thrombin generation measured at low TF is correlated with the risk of recurrent VTE in women.

- The overall rate of recurrence in young and middle aged women is low even in idiopathic cases and it does not outweigh the risk of bleeding on anticoagulant therapy in the majority of the patients.

- Women with hormone-related first VTE seems to have lower risk of recurrence than idiopathic cases but higher risk than women with surgery related first VTE.

8 FUTURE PERSPECTIVES

Further knowledge on the risk factors of VTE and prevention of VTE is needed. Future studies should focus on how VTE can be prevented. Development of clinical scores to estimate the risk of VTE in high risk situations would be one way to do this. In the female population further evaluation of self reported family history as a predictor of VTE could be of interest. It would also be valuable to see if prolonged prophylaxis in surgery/ cast patients with a positive family history would reduce the risk of VTE with acceptable risk of bleeding complications.

Studies on the influence of weight loss on the risk of VTE in obese persons would contribute to further knowledge on how obesity causes VTE. It would also be of clinical use to evaluate if the risk of recurrent VTE is reduced by weight-loss. Obesity is correlated with low grade inflammation in the liver as well as elevated levels of prothrombin. Another condition related to increased levels of prothrombin is non alcoholic fatty liver disease (NFALD). Assessment of NAFLD in obese women with VTE would be a way to further explore the reasons behind the thrombotic effects of obesity.

The risk factors of recurrent VTE need to be better characterized and the reason for the difference in risk between the genders should be explored to improve the clinical care of patients after a first VTE. Clinical decision rules are being developed to estimate the risk of recurrent VTE in patients. This is an important field of research as low risk patients could avoid unnecessary anticoagulant treatment. These scores should be further validated and perhaps separate scores for women should be developed.

9 SAMMANFATTNING PÅ SVENSKA

Venös trombos är ett samlingsnamn för blodproppar i kroppens vener. Hos patienter som drabbas av denna typ av blodpropp sitter proppen vanligen i benets eller bäckenets djupa vener, vilket kallas djup ventrombos (DVT). Blodproppar kan också lossna och färdas med blodet till lungans blodkärl där de fastnar och kallas då lungemboli (LE). Ca 1/1000 personer drabbas årligen av venös trombos. Venös trombos är en sjukdom med många bakomliggande riskfaktorer. Vanligen krävs att patienten har flera riskfaktorer för att drabbas av en blodpropp. Dessa riskfaktorer kan bestå av medfödd benägenhet att lättare bilda blodproppar men också av yttre faktorer som cancersjukdom, fetma, operationer, p-piller och graviditet. Män och kvinnor har delvis olika riskfaktorer för venös trombos där graviditet, p-piller och östrogenbehandling vid övergångsbesvär tillhör de riskfaktorer som huvudsakligen förekommer hos kvinnor. Hos patienter som har fått en venös trombos kommer en andel att få nya episoder med blodpropp. Då LE kan vara en dödlig sjukdom och DVT kan ge långvariga besvär med smärta och svullna ben är det viktigt att undvika återfall i sjukdomen i möjligaste mån. Detta kan göras genom att långtidsbehandla med blodförtunnande läkemedel, men behandling innebär också risk för blödningar som även de kan vara livshotande. Ytterligare kunskap om vilka patienter som har hög respektive låg risk för att återinsjukna i venös trombos kan leda till säkrare behandling och minskad risk för återfall i sjukdomen. Målet med denna avhandling var att utöka kunskapen gällande riskfaktorer för venös trombos hos kvinnor 18-65 år genom att:

1. Studera om uppgiven ärftlighet för venös trombos går att använda för att bedöma risken för att drabbas av blodpropp i samband med p-piller, hormonbehandling och kirurgiska ingrepp.

2. Studera hur fetma hos kvinnor med venös trombos korrelerar med trombingeneration, ett övergripande mått på blodets förmåga att koagulera.

3. Studera om ökad trombingeneration och resistens mot aktiverat protein C är relaterat till ökad risk för återfall i blodpropp.

4. Studera risken för återfall i venös trombos hos kvinnor samt att undersöka om risken för de kvinnor som fått sin första blodpropp i samband med p-piller eller hormonbehandling skiljer sig från de som haft en första blodpropp utan någon utlösande faktor.

1328 kvinnor med DVT eller lungemboli och 1433 friska kvinnor har inkluderats i den nationella studien ”Thrombo Embolism Hormone Study ”(TEHS) år 2003-

2009. Studien handlar om medfödda och förvärvade riskfaktorer för venös trombos hos kvinnor 18-64 år. Samtliga kvinnor har lämnat blodprover för genetisk analys och genomgått en telefonintervju avseende riskfaktorer för venös trombos. En mindre grupp av kvinnorna med blodpropp (244 pers) har också lämnat ytterligare blodprover för mer noggrann analys av olika koagulationsfaktorer och trombingeneration. Dessa ingår i sub-studien ”hypo-TEHS”. Avslutningsvis har alla 1328 patienter med blodpropp följts upp efter i genomsnitt 5 år i ”TEHS follow-up”. Alla återfall i venös trombos under denna tid har registrerats och verifierats.

Sammanfattningsvis visar resultaten av denna avhandling att uppgiven ärftlighet för venös trombos är relaterat till ökad risk för blodpropp hos kvinnor som använder p-piller, hormonbehandling vid övergångsbesvär eller genomgår kirurgi och/ eller gipsbehandling. Detta innebär att man genom att fråga om ärftlighet potentiellt skulle kunna identifiera kvinnor med ökad risk för blodpropp innan man bestämmer om patienten ska ha förebyggande behandling med blodförtunnande medicin efter kirurgi. På samma sätt kan val av preventivmedel och hormonbehandling påverkas kvinnans risk för att utveckla blodpropp. Vidare finner vi att en ökad benägenhet för blodet att koagulera mätt med trombingeneration är korrelerat till en ökad risk för återfall i blodpropp. Även APC-resistens utan förekomst av genetiska rubbningar som faktor V Leiden är relaterat till ökad risk för återfall. Hos kvinnor med venös blodpropp visar resultaten att fetma är relaterat till ökad inflammation och trombingeneration vilket kan vara en förklaring till varför kvinnor med fetma har ökad risk för både förstagångsinsjuknande och återfall i blodpropp. Gällande den totala risken för återfall i venös trombos hos kvinnor i åldern 18-65 år finner vi att denna generellt är låg. Hos de allra flesta kvinnor i denna åldersgrupp är risken så låg att den understiger risken för allvarliga blödningskomplikationer med långtidsbehandling med blodförtunnande läkemedel. Återfallsrisken är högst hos patienter där ingen säker riskfaktor för venös trombos har kunnat identifieras medan den är lägst för patienter som fått sin blodpropp i samband med ett kirurgiskt ingrepp. Risken för återfall hos kvinnor som fått sin första blodpropp i samband med p-piller eller hormonbehandling har en risk för återfall som ligger någonstans mitt emellan de ovan nämnda.

10 ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the help and encouragement of a number of people during the past ten years.

First, and most of all, I would like to thank my main supervisor Gärd Lärfars. Besides from being one of the most competent doctors I know in the field of coagulation you also have a fantastic way of always making me feel that our research is interesting and important. No matter how negative I feel before a meeting with you, I always come out with new energy and ideas. Thank you for all your support and time over the years.

Johanna Adami, my co-supervisor who knows all there is to know about epidemiology. You have a fantastic ability to encourage people and you are always bringing the best out of me. Thank you for pep talks in nice restaurants, wine on the balcony and hyper-effective revisions of manuscripts.

Margareta Holmström, my other co-supervisor and head of the Department of Coagulation in Karolinska University Hospital. Thank you for your skillful help through the whole process of my PhD studies and for your valuable contributions to several of the manuscripts.

I would also like to thank:

Helle Kieler, co-author, and principal investigator for the TEHS-study. Thank you for all your support, patience and expert help during all this time.

Svetlana Tchaikovski and Jan Rosing, co-authors of paper II and III. Thank you for introducing me to thrombin generation and for your patience, encouragement and help with the manuscripts. I have also enjoyed our interesting discussions at meetings in different parts of the world.

Katarina Bremme, co-author of paper II and III, for all the interesting discussions, thoughtful comments and great engagement in the hypo-TEHS study.

Jovan Antovic, co-author of paper II, for all your help with thrombin generation measurements with the Innovance ETP-assay and work with the manuscript.

Tomas Lindahl, for your engagement in planning hypo-TEHS and for organizing the measurements of coagulation factors.

Annika Åkerström, Elisabet Stjernberg and Ebba Hallberg for all the work with the interviews and co-ordination of TEHS. Sofia Olsson, Annika Ahlén and Doris Näslin for all the work with hypo-TEHS.

Annica Bergendal and Maria Ljungqvist, probably the best fellow PhD-students in the world. Who needs therapy when there are friends and colleagues like you?

My present and former co-workers at the section of hematology and coagulation at the Department of Internal Medicine at Södersjukhuset; Maria, Micke, Patrik, Eva, Birgitta, Lars-Göran, Johan, Mats and Janne for creating a research friendly environment and for taking care of my patients when I was not around.

Fellow students of the research school in epidemiology at Karolinska Institutet. For all the interesting discussions, critical reviews and the fun parties.

Lina Benson, for statistical help and discussions at all times.

My fantastic friends Lisen, Petra, Maria C, Anneli, Emma, Marja, Maria L, Karin and all the rest for always being there and helping me realize what is really important in life.

My large family with my mother Lena and Lars, my father Claes and Bodil, my siblings Jocke, Erika, Fabian and of course my sister Linda who is the most extraordinary sister anyone could have.

My grandmother Birgitta Sonnevi who has been one of my greatest supporters since I was born, I'm so happy to share this with you.

And last but not least, the most precious persons in my life, my incredible children Elias and Hanna and the man who have put up with me and stood by my side for more than 20 years, Fredrik. I love you all more than I can express.

This thesis was financially supported by unrestricted research grants from Astra-Zeneca, Leo-Pharma, Janssen-Cilag, Novartis, Organon, Schering,Wyeth, AFA research foundation , and Stockholm Count Council Research Found (ALF).

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