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NEW DIAGNOSTIC APPROACHES FOR VENOUS THROMBOEMOBLISM AND THROMBOPHILIA

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“Everything not saved will be lost”

Nintendo
Quit Screen

Till Erik, Calle, Oscar och Mamma

NEW DIAGNOSTIC APPROACHES FOR VENOUS THROMBOEMOBLISM AND THROMBOPHILIA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

Background

Venous thromboembolism is a serious disease comprising both pulmonary embolism and deep vein thrombosis. Venous thromboembolism causes considerable mortality and morbidity, with residual symptoms such as pulmonary hypertension and post thrombotic syndrome. Current assays for venous thromboembolism and the predisposition to develop venous thromboembolisms (thrombophilia) can only reflect a fragment of the complicated hemostatic processes. This thesis evaluates the usefulness of current and emerging hemostatic assays that reflect several aspects of the hemostatic process. Increased diagnostic specificity would enable better diagnosis and risk stratification of patients with venous thromboembolism. Improved biomarkers for venous thromboembolism and thrombophilia have the potential to prevent major morbidity and mortality by guiding treatment and prophylaxis to the right patients at the right time.

Aim

The aim of this project was to investigate assays that could improve the care for patients with suspected venous thromboembolism or thrombophilia. We evaluated the usefulness of current and emerging biomarkers for venous thromboembolism, explored the utility of emerging biomarkers for thrombophilia and functionally characterized a novel prothrombotic genetic variant.

Methods

A prospective case-control study of 954 patients with clinically suspected acute deep venous thrombosis or pulmonary embolism were recruited from the emergency department and analyzed by four D-dimer assays, fibrin monomers, thrombin generation and fibrin aggregation assays. The discriminatory accuracy of all assays and of age-adjusted cutoffs for D-dimer was evaluated.

From the special coagulation laboratory, we included 369 patients with clinical criteria for thrombophilia testing. Plasma and DNA samples were analyzed by the global hemostatic assays Overall Hemostatic Potential (OHP) and Endogenous Thrombin Potential (ETP) as well as genotyped for several prothrombotic variants.

In a separate study, a novel genetic variant with possible prothrombotic effect was characterized by the same assays, prothrombin levels, mRNA expression, and scanning electron microscopy of fibrin clot structure.

Conclusion

Use of age-adjusted cutoffs for D-dimer could lead to a >5% decrease in false positivity rate and in elderly patients this avoidance of unwarranted radiology could even affect as many as 20% of patients with suspected venous thromboembolism. The Overall Hemostatic Potential, Endogenous Thrombin Potential and fibrin monomer assay were not superior to D-dimer for diagnosis of deep venous thrombosis or pulmonary embolism. Global hemostatic assays and extended investigation of prothrombotic genetic variants discretely improved the predictive ability of the classical genetic thrombophilia markers and the proportion of patients with verifiable hypercoagulability. We could also suggest a connection between increased thrombotic risk and a recently discovered synonymous single nucleotide polymorphism.

SVENSK INTRODUKTION

Venösa blodproppar drabbar mer än 20 000 personer årligen i Sverige, varav 25% av patienterna får kvarstående besvär. Vanligen drabbas benets djupa vener eller venerna i lungan, med bensvullnad respektive andnöd som huvudsymtom. Blodpropp i lungan är en akut och allvarlig, i vissa fall även livshotande sjukdom. Blodproppar kan bero på yttre orsaker som benbrott eller graviditet. De kan också bero på skillnader i blodets sammansättning, som gör att vissa har lättare att få blodpropp än andra.

Läkare som misstänker venösa blodproppar har få användbara biomarkörer till sin hjälp, främst mäts D-dimer i blodprov. Tyvärr erhålls opålitliga provsvar om patienten har vissa andra sjukdomar eller andra tillstånd samtidigt. Exempelvis störs analysen av infektioner, inflammation, cancer och normala tillstånd som graviditet och stigande ålder. På akutmottagningen tvingas man därför ofta till att utreda misstänkta venösa blodproppar med speciella röntgenundersökningar med långa väntetider och viss risk för patientskada ifrån strålning och kontrastmedel.

Att bedöma risknivån för att få framtida venösa blodproppar är också ett område där det saknas optimala biomarkörer. Om riskbedömningen skulle kunna stöttas genom bättre markörer för propprisk, så skulle det öka sannolikheten för att rätt patienter skulle ordineras blodförtunnande mediciner. Förhoppningsvis skulle detta kunna minska både onödiga blödningsrisker och risken för allvarliga proppar.

Denna avhandling har undersökt sätt att förbättra biomarkörer för diagnos av blodpropp och blodproppsrisk. Vi har gjort det genom att utvärdera utvecklingar av dagens biomarkörer och även lovande nya markörer. Vi har också undersökt en nyligen upptäckt mutation, som tycks vara kopplad till ökad propprisk, för att klargöra dess verkningsmekanism.

I den här avhandlingen har vi samarbetat mellan olika kliniker och sjukhus, samt med diagnostikaindustri och forskningslaboratorier i Sverige och Europa. I utvärderingen har vi utnyttjat den stora mängd blodprover som passerat Stockholms sjukhuslaboratorier och använt överblivet provmaterial som annars skulle ha kasserats. Trots att vi haft varierande framgång med att hitta bra biomarkörer, är min förhoppning att våra framsteg ska bidra till att förbättra människors hälsa i närtid och på sikt.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications, which are referred to in the text by their roman numerals (Study I-IV)

I. **Farm M**, Siddiqui AJ, Onelöv L, Järnberg I, Eintrei J, Maskovic F, Kallner A, Holmström M, Antovic JP
Age-adjusted D-dimer cut-off leads to more efficient diagnosis of venous thromboembolism in the emergency department: a comparison of four assays.
Journal of Thrombosis and Haemostasis 2018; 16: 866–75.

II. **Farm M**, Antovic A, Schmidt DE, Bark N, Soutari N, Siddiqui AJ, Holmström M, Pruner I, Antovic JP
Diagnostic accuracy in acute venous thromboembolism: comparing D-dimer, thrombin generation, overall hemostatic potential and fibrin monomers.
Thrombosis Haemostasis Open, in revision.

III. **Farm M**, Schmidt DE, Onelöv L, Zong Y, Antovic A, Fahlén A, Soutari N, Bark N, Pruner I, Antovic JP
Thrombin generation and fibrin aggregation in clinically suspected thrombophilia.
Manuscript.

IV. Pruner I, **Farm M**, Tomic B, Gvozdenov M, Kovac M, Miljic P, Soutari N, Antovic A, Radojkovic D, Antovic JP, Djordjevic V
The silence speaks, but we do not listen: synonymous c.1824C>T gene variant in the last exon of the prothrombin gene as a new prothrombotic risk factor
Clinical Chemistry, 2020, 66, 379-389

V. Salas E*, **Farm M***, Pich S, Onelöv L, Guillen K, Ortega I, Antovic JP, Soria JM
Predictive Ability of Genetic Risk Scores for Venous Thromboembolism Is Similar in Northern and Southern Europe.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

% Neg	percentage of negative results
% FP	percentage of false positive results
α2M	α2-macro globulin
APC	Activated Protein C
aPTT	Activated Partial Thromboplastin Time
BMI	Body mass index, kg/m^2
CAT	Calibrated Automated Thrombogram
CI	Confidence Interval, most commonly 95% confidence intervals
CLSI	Clinical and Laboratory Standards Institute
CLT	Clot Lysis Time
CRP	C-reactive protein, biomarker of inflammation
CTI	Corn Trypsin Inhibitor
CTPA	Computed tomography pulmonary angiogram
CUS	Compression ultrasound
CVI	Cerebrovascular insult
D-Di	D-dimer
D-DU	D-dimer Equivalent Units, D-dimer assay calibration
DNA	Deoxyribonucleic acid, genetic code
dRVVT	Diluted Russel Viper Venom Time
DVT	Deep Venous Thrombosis
EAF	Effect Allele Frequency
ELISA	Enzyme-Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
ETP AUC	ETP; Area under the thrombin generation curve
ETP C_{Max}	ETP; peak thrombin concentration
ETP T_{max}	ETP; time required to reach C_{max}
ETP T_{lag}	ETP; time to the start of thrombin generation
F11	Gene for coagulation Factor 11
F2	Gene for prothrombin, coagulation factor II
FII	Prothrombin, coagulation factor II
FII c.1824C>T	Polymorphism in the *F2* gene, *F2* rs3136532
FII c.20210G>A	Polymorphism in the *F2* gene, *F2* rs1799963
FEU	Fibrinogen Equivalent Units, D-dimer assay calibration
FGG	Gene for the fibrinogen gamma chain
FM	Fibrin Monomers

FV	Coagulation factor V; coagulation factors abbreviated F + roman numeral
FVa	Activated coagulation factor V; [***a***] signifies an activated coagulation factor
FVL	Factor V Leiden mutation, *F5*, rs6025
GHA	Global Hemostatic Assay
GRS	Genetic Risk Score
GWAS	Genome-wide association study
Hmz	Homozygous
HR	Hazard Ratio
Htz	Heterozygous
IQR	Inter-quartile range
NNT	Numbers needed to test
NPV	Negative predictive value
OCP	Overall Coagulation Potential
OFP	Overall Fibrinolysis Potential
OHP	Overall Hemostasis Potential
OR	Odds Ratio
P	Proportion
P-value	Probability value, p-values <0.05 were considered significant
PE	Pulmonary Embolism
PPP	Platelet Poor Plasma
PPV	Positive predictive value
PT (INR)	Prothrombin Time (International Normalized Ratio)
RFLP	Restriction fragment length polymorphism
ROC	Receiver Operator Characteristic
ROC AUC	Area Under the ROC-curve
RNA	Ribonucleic acid
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
ssPE	Subsegmental Pulmonary Embolism
TF	Tissue Factor, Coagulation Factor III
TGA	Thrombin generation assay
TiC	ThromboInCode®
tPA	Tissue-type Plasminogen Activator
VTE	Venous Thromboembolism
vWF	von Willebrand factor
ZPI	Protein Z-dependent protease inhibitor

1 BACKGROUND

1.1 VENOUS THROMBOEMBOLISM

1.1.1 Morbidity and mortality

Venous thromboembolism (VTE) is the third most common cardiovascular disease [1] and a cause of substantial morbidity and mortality worldwide. VTE is estimated to be the leading cause of preventable hospital mortality [2] and causes a considerable health economic cost [3]. However, even though verified VTE is treatable and most prophylactic treatment options are well-tolerated [4], both VTE and thrombophilia are under-diagnosed and under-treated [5-7]. The problem could be reduced by treatment and selective prophylaxis in adequate time [4, 8].

VTE is constituted primarily by deep venous thrombosis (DVT, 2/3) and pulmonary embolism (PE, 1/3) [5], Figure 1. More uncommon sites are portal vein thrombosis, upper extremity deep vein thrombosis, mesenteric vein thrombosis, renal vein thrombosis and cerebral venous sinus thrombosis.

VTE occurs in 2-3/1000 individuals, with an incidence in adolescence of 0.1/1000, increasing to 8/1000 above 80 years of age [9]. Women are subject to VTEs earlier in life than men, explained by the fact that they are provoked by hormone-based contraceptives and pregnancy [7, 10], but there is no difference in overall incidence between men and women. Men have a 75% higher risk of a recurrent VTE after a first episode [11] and the age-related increase in VTE begins by the age of 50 in men and 60 in women [7].

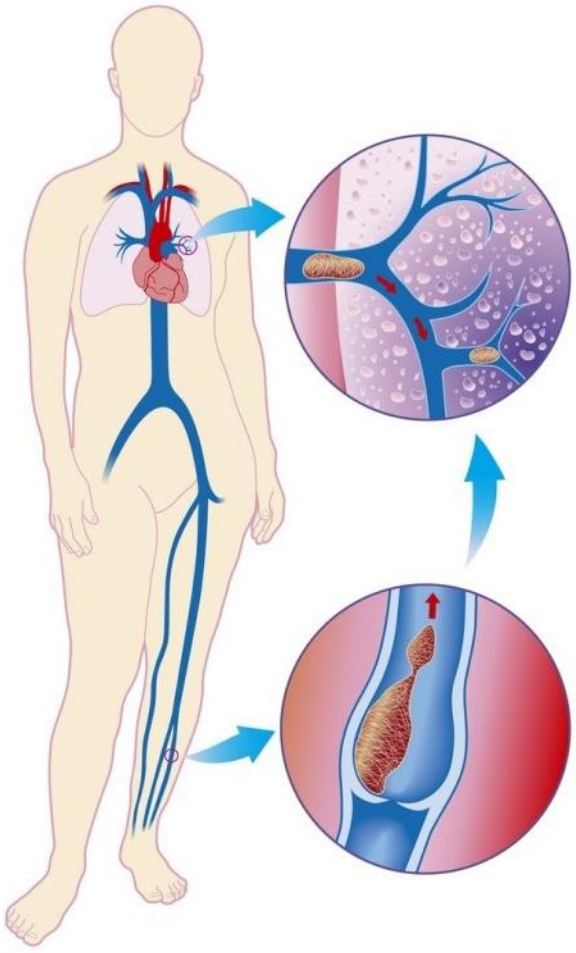


Figure 1. *DVT in leg embolizing to the lungs and becoming a pulmonary embolism. Image: David Gifford, Science photo library*

The most frequent symptoms at diagnosis of DVT are pain (82.8 %) and swelling of extremities (73.3 %). The corresponding symptoms of PE are dyspnea (75.6 %) and chest pain (45.5 %) [1]. Aside from acute symptoms, VTE causes considerable long-term morbidity with 25% of patients developing residual symptoms such as post-thrombotic syndrome with ulcerations and chronic swollenness and chronic pulmonary hypertension [3]. VTE is considered a chronic disease, because of the highly prevalent residual symptoms and the permanent increased risk of recurrent venous thrombosis [5], with a cumulative recurrence rate after ten years of about 30% [12]. VTE is also the major cause of lost disability-adjusted life years (DALYs) in both high and low-income countries [13].

In addition to the considerable morbidity of VTE, the thirty-day all-cause mortality of pulmonary embolism is approximately 12% and DVT 6% [10]. An estimated 12% of all deaths in Europe are caused by PE [5]. Fatal PEs are only diagnosed previous to death in 7% of cases, the majority of fatal events are PE that have remained undiagnosed and sudden fatal PE, with 59% and 34% respectively [5]. The morbidity and mortality of VTE could be significantly reduced by prevention, early correct diagnosis and appropriate treatment [4, 8].

1.1.2 Classification of deep venous thrombosis and pulmonary embolism

PE is divided into segmental PE and subsegmental PE (ssPE), determined by imaging techniques. DVT the leg is classified as distal or proximal; where distal DVT is located below the popliteal vein. The subset of distal DVTs that are isolated to the muscular veins of the calf, and do not extend into the deep venous system (i.e. soleus, gastrocnemius) are classified as muscle vein thromboses. Distal DVT if left untreated, can be expected to eventually extend into a proximal DVT and possibly further into PE in about 15% of cases[4].

Symptoms of VTE are related to localization and size, distal DVT and ssPEs are to a higher extent without symptoms and/or critical clinical complications. Subsequently, whether or not to treat smaller VTEs is a challenging decision, given the bleeding risk of anticoagulant therapy. In Sweden, isolated distal DVTs are treated for four weeks to six months, primarily based on symptoms, VTE size and risk factors for recurrent VTE. However, many guidelines maintain that patients with small VTE may have a negative risk-benefit-ratio from anticoagulant treatment. The recent CHEST* guidelines for antithrombotic therapy for VTE recommend surveillance over treatment in distal DVT and ssPE, unless the patient has serious symptoms or risk factors [4].

* *CHEST; the official publication of the American College of Chest Physicians*

1.2 PATHOGENESIS OF VENOUS THROMBOEMBOLISM

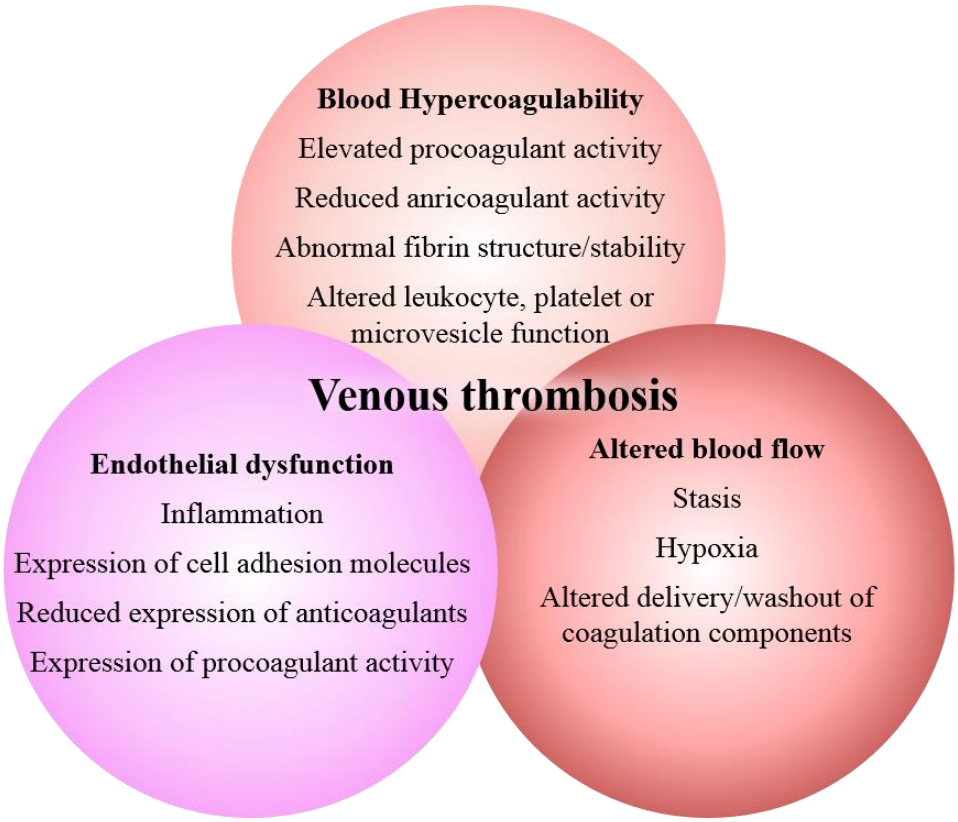


Figure 2. *Modern rendition of Virchow's triad, adapted from Byrnes et al.* [14].

1.2.1 Virchow's Triad

Venous thrombosis has classically been described as a combined effect of prothrombotic changes in the blood flow, vascular wall and blood composition, i.e. Virchow's Triad [15], Figure 2. The model is as relevant as ever. Blood composition would currently be considered to encompass the individual concentrations of pro- and anticoagulant proteins and hematologic cells as well as cell derived microvesicles. Reduced blood flow or stasis is still highly relevant in VTE, because venous clots are formed under low shear stress in contrast to arterial clots [16, 17]. Reduced flow or static blood in the valve pocket sinuses decreases the inhibiting regulation that wall shear stress normally has on the endothelial lining of the blood vessel and leads to initiation of thrombus formation [18]. The endothelial cells of the vessel walls have been demonstrated as important in formation of both venous and arterial thrombi. Although, in venous thrombosis formation, the endothelial lining is mainly intact, unlike in arterial thrombosis formation [17].

Contemporary concepts that complement Virchow's Triad, are the cell-based model of hemostasis, which describes how essential the activated platelets are for the plasma coagulation [19] and the intimate interactions of platelets and coagulation factors in VTE [20].

1.2.2 Endothelial Cells

An important initiator of VTE formation is the activation of endothelial cells, primarily caused by the hypoxia that may arise in the valve pocket sinuses, or possibly by inflammation. Activation leads to expression of adhesion receptors to the cell surfaces, facilitating binding of circulating leukocytes and microvesicles. Subsequent activation of monocytes induces expression and de-encryption of tissue factor (TF) [21]. TF is a potent procoagulant surface antigen, constitutively expressed on the cells that form an anti-hemorrhagic envelope around blood vessels, especially in critical organs such as the brain, heart and lungs. Expression of TF can also be induced in activated endothelial cells and monocytes. TF is additionally present on monocyte derived microvesicles and possibly on neutrophils, eosinophils and T-cells and activated platelets with derived microvesicles, although this is controversial [21], Figure 3.

Activated monocytes with increased expression of TF are most probably an important driving factor behind the increased risk for VTE in sepsis, cancer and after surgery [17, 21].

1.2.3 Coagulation Cascade

TF in complex with activate Factor VII (FVIIa) initiates coagulation and induces thrombus formation. The primary source of FVIIa needed for the initiation of coagulation is thought to be the minute amounts of free FVIIa present in the circulation. The complex of TF-FVIIa, the extrinsic tenase complex, activates small amounts of Factor X and Factor IX (which is the primary substrate of the complex). Activated Factor Xa (FXa), adsorbed on the cell surface, can convert some prothrombin into cell-bound meizothrombin, which can activate some Factor V (FV), released by activated platelets. Activated FV (FVa) binds to FXa forming the prothrombinase complex, which activates small amounts of prothrombin into thrombin. The initiation phase of the coagulation is concluded when thrombin activates the cofactors Factor V and Factor VIII as well as Factor XI, Factor VII, Factor XIII and platelets.

The prothrombinase complex and the intrinsic tenase complex, a complex of FVIIIa-FIXa, assemble on the negative surfaces of activated platelets. This increases their enzymatic efficiency manifold leading to the generation of large amounts of FXa and thrombin [22]. The propagation of the coagulation cascade is additionally driven by the activation of FIX by FXIa, generated in the initiation phase. Thrombin also initially feed-back stimulates its own generation by activating more FVII, although the TF-FVIIa-complex is quickly inhibited by Tissue Factor Pathway Inhibitor (TFPI) after the initiation of coagulation [21].

Factor XIIa (FXIIa) is another driver of thrombus formation and is considered by some to play a part in the formation of pathological VTE associated with immunothrombosis and cancer. After activation by negative surfaces, such as extracellular nucleic acids or polyphosphates from activated platelets or bacteria [23], FXIIa activates Factor XI and thereby the intrinsic path of the coagulation cascade, see Figure 3.

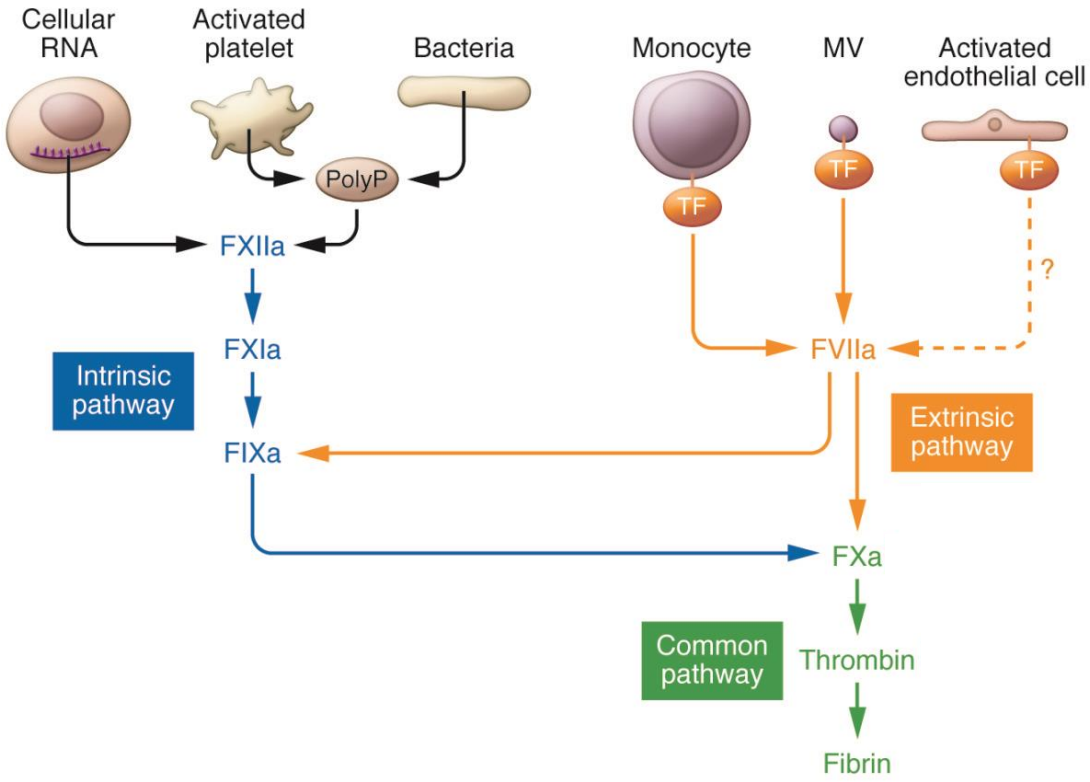


Figure 3. *Pathological activation of the extrinsic pathway via Tissue Factor (TF) expression in activated monocytes, cell derived micro vesicles (MV) and activated endothelial cells. Cellular RNA and polyphosphate (PolyP) released from activated platelets or bacteria activate FXIIa in the intrinsic pathway. Mackman* [17].

1.2.4 Platelets

It is now clear that activated platelets have a role not only in the primary, but also in the secondary hemostasis, primarily because they provide the negative cell surfaces where the coagulation process is localized, which both enhances the thrombin generation, protects the coagulation enzymes from inhibition and regulates that coagulation is not disseminated in the circulation. Platelets are activated by the exposure of subendothelial collagen in hemostasis, but can also be activated by thrombin via activation of protease-activated receptors on the platelet cell membrane, a pathway that is active in both hemostasis and VTE formation [20]. Activated platelets also contribute activating factors to many steps of the coagulation process. Upon activation and degranulation, the content of the α-granules is

released, which contains a large amount of different proteins, some of which are coagulation factors; fibrinogen (endocytosed from blood), platelet FV, FXIII, Ca^{2+} (i.e. Coagulation Factor IV), von Willebrand factor as well as FVIII, FIX and FXI according to some reports [22]. Procoagulant platelet polyphosphates are also released at the degranulation of the dense granulae of the platelets. The mechanism for their procoagulant activity is still controversial, but they have been claimed to directly activate FXII [24-26]. As mentioned before it is also controversial whether activated platelets and platelet derived microvesicles are a source of intravascular, cell membrane bound TF [21].

1.2.5 Fibrinogen and fibrin degradation products

Thrombin is the key enzyme in the coagulation because it is a platelet agonist, activates several coagulation factors, FV, FVII, FVIII, FXI and FXIII, and not least because it converts fibrinogen into fibrin in the final step of the coagulation cascade. The fibrin monomers (FM) then self-polymerize into insoluble fibrils. The fibrils are cross-linked into fibrin networks by the transglutaminase Factor XIIIa.

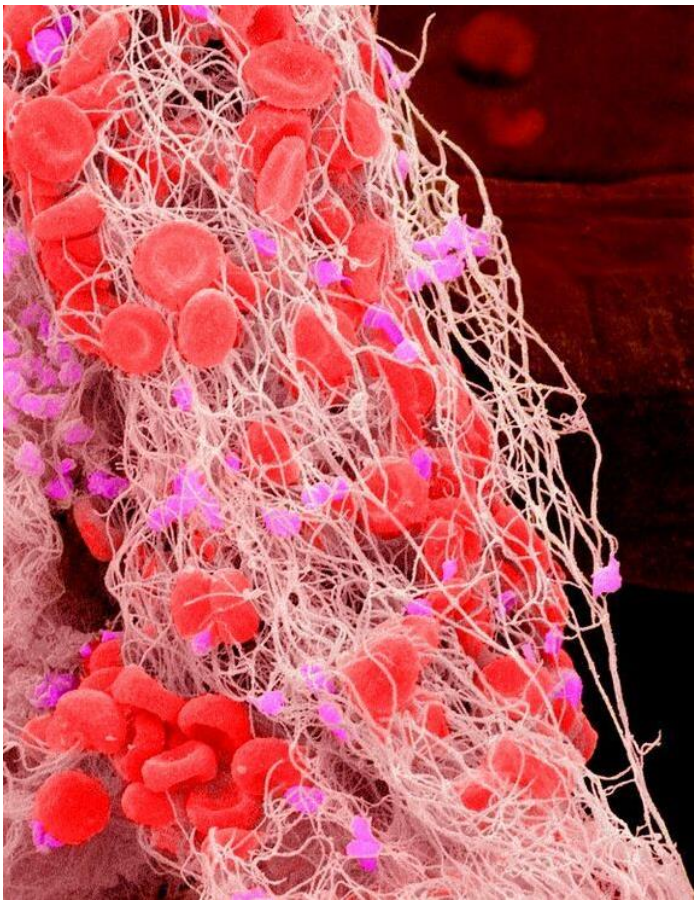


Figure 4. *Blood clot visualized by SEM.*
Red; erythrocytes, white; fibrin, pink; platelets.
Image: Susumu Nishinaga, Science photo library.

The structure of the fibrin clot will determine the resistance to fibrinolysis [27]; tight fibrin structures with thin fibers, increased number of branch points and small pores result in a slower fibrinolysis rate than a loose structure of thick and unbranched fibers. Clot structure is affected by thrombin concentration, and to a lesser extent by pH, ionic strength, Ca^{2+}, fibrinogen concentration and FXIII [28]. In fibrinolysis, degradation products are released into plasma, some carrying the epitope of two adjacent D moieties of cross-linked fibrin, known as D-dimers.

1.3 DIAGNOSIS OF VENOUS THROMBOEMBOLISM

1.3.1 Clinical prediction rules for venous thromboembolism

DVT and PE can present with very discrete symptoms, or with symptoms similar to other conditions. A definite diagnosis of DVT and/or PE can generally not be achieved by only clinical examination of the patient, and the quality of laboratory assays and imaging techniques are of utmost importance. Diagnosis of VTE is based on a sequential approach following a clinical pre-test probability assessment (Figure 5). In patients with low pre-test probability for VTE, D-dimer is analyzed in plasma to potentially exclude VTE, if negative. Mid/high pre-test probability leads to imaging that can verify or rule out DVT or PE [29]. Clinical pre-test probability assessment is most often done by the Wells clinical prediction rule for DVT and by the Wells rule for PE [29] or the Geneva rule. The Wells and Geneva rules for PE have similar accuracy, based on a systematic meta-analysis [30] and a direct prospective comparison [31].

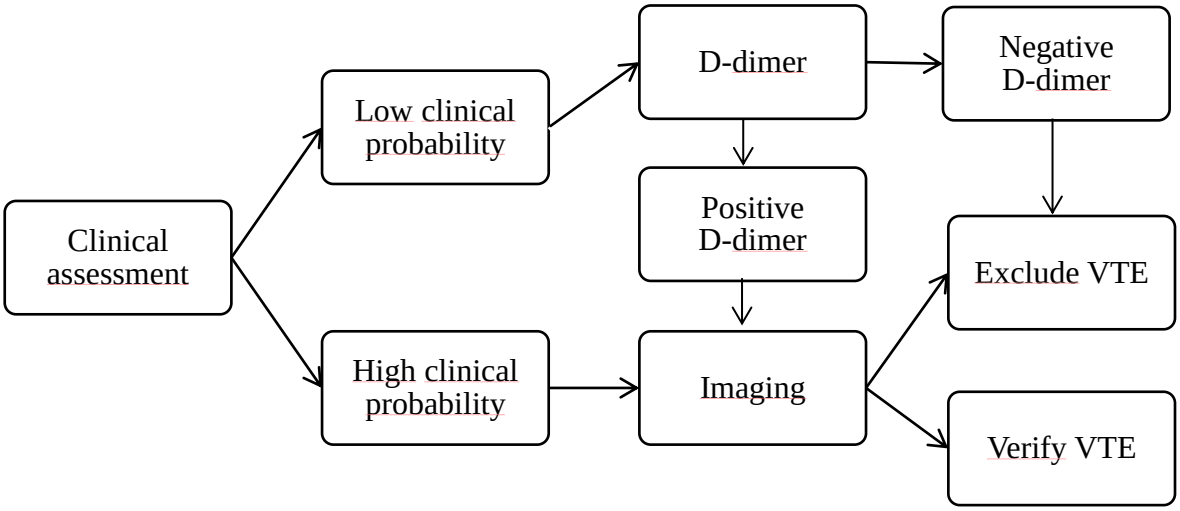


Figure 5. *Diagnostic algorithm for DVT and Pulmonary Embolism in the emergency department, Socialstyrelsen* [32].

1.3.2 Diagnosis of deep venous thrombosis in the leg

DVT can be verified by venography of the lower extremity or whole-leg echo-color-doppler ultrasonography. Venous 2-point compression ultrasonography (CUS), with compression at the inguinal and the popliteal vein can also be used to verify DVT. The Gold Standard is venography, which is however associated with exposure to radiation and intravenous contrast, both of which can have adverse iatrogenic effects [33, 34].

Many guidelines recommend the two-point CUS, the CHEST* guidelines even discourage the use of whole leg examination [4]. There are practical and economical motives to these recommendations, based on the increased simplicity and repeatability of the 2-point CUS.

The clinical importance of diagnosing distal DVT and muscle vein thrombosis is also highly debatable, as discussed in section 1.2.2. A meta-analysis of the accuracy of non-invasive diagnosis of DVT calculated the weighted mean sensitivity of CUS to detect symptomatic proximal DVT to 94% [95% confidence interval (CI) 92–95%] with a specificity of 98% [95% CI 97-98%]. The sensitivity for detecting distal proximal DVTs was 91% [95% CI 82-96%] and the specificity 99% [95% CI 97-100%] [35]. CUS has also been shown in one randomized controlled trial (RCT) to be clinically equivalent to whole leg ECD, when used for the management of symptomatic outpatients with suspected DVT of the lower extremities [36]. This has been interpreted by many in the field as corroborating the theory that distal DVTs are clinically irrelevant, as distal DVTs can only be visualized by venography or Duplex-doppler ultrasonography [36].

1.3.3 Verifying pulmonary embolism

Computed tomography pulmonary angiography (CTPA) is the preferred technique for verifying PE, although it is associated with radiation and intravenous contrast medium with risk for adverse effects [33, 34]. Patients that cannot be exposed to this risk, are referred to pulmonary ventilation-perfusion scintigraphy, however associated with the risk of intravenous isotopes [35].

Since the introduction of CTPA for diagnosing PE, the frequency of ssPE has increased from approximately 5% to more than 10% of PEs [1]. The apparent incidence of PE has also increased by 80% during the same period, without a significant decrease in mortality following the supposed increased proportion of patients that received anticoagulant treatment [37]. This notable dissonance is another argument for the stance that ssPE are clinically irrelevant and should generally not receive anticoagulation.

1.3.4 D-dimer as a biomarker of venous thromboembolism

While imaging techniques are important to verify VTE, the use of biomarkers in selected cases has the advantage of reduced cost, time and iatrogenic complications. D-dimer in plasma reflects the recent activation of both hemostasis and fibrinolysis, because they are the degradation products of cross-linked polymerized fibrin. D-dimer assays are immunochemical, using antibodies directed against the epitope of two interconnected terminal D-subunits of fibrinogen, Figure 6.

** CHEST; the official publication of the American College of Chest Physicians*

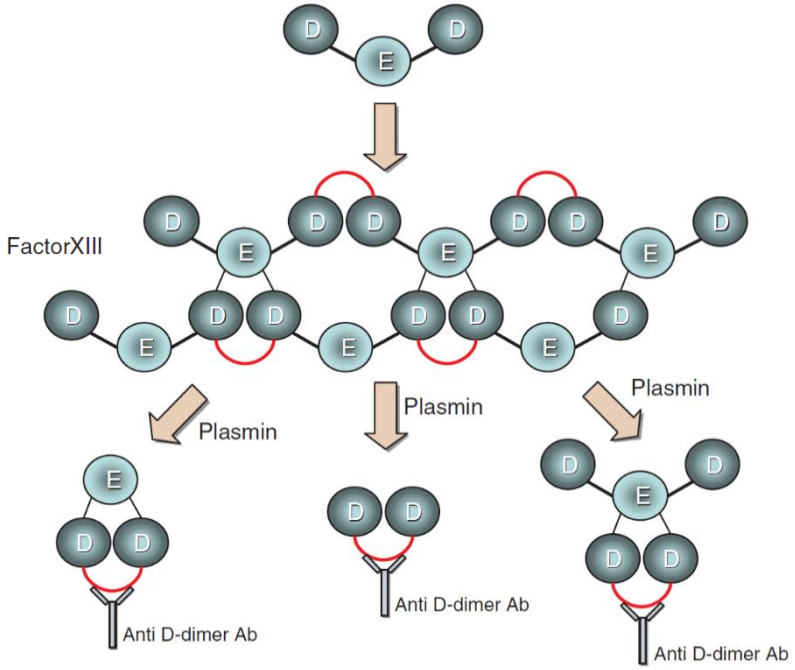


Figure 6. *Formation and measurement of D-dimer, adapted from Lippi et al* [38].

Initially, D-dimer was analyzed by ELISA methods, but high-throughput nephelometry or particle-enhanced turbidimetry are now standard. The quantitative results cannot easily be related between assays. The poor analytical comparability is due to size heterogeneity of the D-Dimer fragments, and that assays have different reactivity for the various fragment lengths [39, 40]. There is presently no international reference calibrator for D-dimer because the balance of different fragment sizes varies inter-individually as well as with clinical conditions [41]. Conditions with an interrupted blood flow, such as thromboembolism, tend to produce shorter fragments whereas conditions with a maintained flow such as disseminated intravascular coagulation (DIC) generally produces longer fragments [39]. To improve the comparability of D-dimer, results are preferably reported in mg/L Fibrinogen Equivalent Units (FEU) or mg/L D-dimer Equivalent Units (D-DU) [38].

D-dimer assays generally have a high diagnostic sensitivity for VTE, but specificity for VTE is low due to other causes of fibrin strand formation. D-dimer is formed in several other conditions than fibrin aggregation in thrombosis[42], such as pregnancy, cancer, trauma, inflammation, infection and advanced age [43]. Many of these conditions are associated with an increased risk of venous thrombosis and they are frequently encountered in VTE-patients. The clinical implications are extensive, particularly in patients with comorbidities or advanced age. Despite the low specificity, D-dimer is the only biomarker in common clinical use for diagnosis of VTE [38].

Since false positive results are so common, D-dimer has been determined to be clinically valuable only in cases with low pre-test probability. The recommendation is to exclusively use D-dimer to rule out PE and DVT of the lower extremities in patients with a low clinical probability [29, 38]. To optimize this functionality, D-dimer cutoffs are set at a level where sensitivity is maximal. The Clinical and Laboratory Standards Institute (CLSI) recommends that cutoff be set so that sensitivity is ≥ 97%, with lower limit of 95% confidence interval [95% CI] of ≥ 90% and negative predictive value (NPV) ≥ 98% with lower limit of 95% CI of ≥ 95%). The recommendation regards D-dimer analysis to rule out VTE in a clinical population with low or intermediate pretest probability of VTE [44]. It is not defined whether the high sensitivity includes exclusion of distal DVT and ssPE, which are associated with lower D-dimer concentrations and a higher degree of false negative results [45].

Both the prevalence of VTE and the plasma concentration of D-dimer increase with age. Even in a healthy population of ≥70-year old's, 50% have a D-dimer concentration above the cut-off [46]. Some advocate that D-dimer testing should be entirely avoided in the elderly [46, 47], but the effect of this approach would be that patients had to undergo imaging techniques instead, even when the probability of VTE was low. Age-adjusted cutoffs have been validated and recommended in DVT and PE, to increase specificity and markedly decrease the rate of false positive results in older patients while preserving sensitivity [48-52]. Some clinical guidelines already recommend the use of age-adjusted cut-off values [37, 53, 54], using the formula originally suggested by Douma et al. in 2010 [55]. However the opinion has been questioned [56-58], and a recent Cochrane review of D-Dimer to rule out pulmonary embolism did not find enough evidence to recommend age-adjusted cutoffs [59].

1.3.5 Fibrin monomers and additional biomarkers of venous thromboembolism

Another marker of activated coagulation which can be of use in acute thrombosis is fibrin that has not yet polymerized, as measured in the assays Fibrin Monomers (FM) and Soluble Fibrin [60, 61]. The latter is comprised of different lengths of still soluble oligomerized fibrin, whereas FM is a more homogenous measurand. Both assays are immunochemical analyses by ELISA or particle enhanced turbidimetry.

Concentrations are demonstrably low in normal plasma, and increase faster than D-dimer in thrombotic disease [62]. However, it is important to bear in mind that anticoagulant therapy rapidly normalizes the amount of circulating FM [63]. Soluble fibrin and FM could be alternatives to D-dimer to determine acute VTE, if the diagnostic sensitivity was also sufficient. FM or soluble fibrin could therwisepossibly improve diagnosis if used together

with D-dimer for the exclusion of VTE [64]. To date, the high variability between assays has been part of retarding the broad clinical implementation of these assays. Possibly, an alternative would be to analyze FM together with D-Dimer to counteract the lower sensitivity of FM [63].

Another marker of venous thrombosis, but also of ongoing thrombin generation *in vivo*, is the prothrombin fragment 1+2 (F1+2) analyzed in plasma or urine. F1+2 is a fragment of the prothrombin molecule with a half-life of approximately 90 minutes, that is discharged when prothrombin is cleaved into thrombin by the prothrombinase complex. F1+2 has been utilized to assess thrombotic risk and monitor anticoagulant therapy but is also elevated in acute VTE and in the same common conditions that increase the D-dimer. In comparisons with D-dimer, F1+2 has displayed less diagnostic sensitivity and inferior diagnostic ability by receiver operating characteristic (ROC) [65]. Another thrombin related marker that has gone out of vogue is the thrombin-antithrombin complex, which could also be used as a less sensitive than D-dimer marker of acute VTE. The complex is formed when the coagulation cascade is inhibited as thrombin cleaves antithrombin and forms a covalent 1:1 thrombin-antithrombin complex, that is rapidly cleared from circulation, with a half-life of 15 min [65]. Both F1+2 and the thrombin-antithrombin complex are now primarily biomarkers of *in vivo* thrombin generation and neither was established as a clinical routine marker.

Several other biomarkers have been investigated for the diagnosis of acute VTE, for example CRP, neutrophil-lymphocyte ratio [66], mean platelet volume [66], p-selectin [67], Protein S, APC-PC inhibitor complex, coagulation Factor VIII, von Willebrand factor, platelet derived TF-bearing microvesicles [68] and markers of neutrophil extracellular traps [69].

1.3.6 Clinical risk factors for venous thromboembolism

Patients with increased risk for a first or recurrent VTE event may be indefinitely treated with prophylactic anticoagulants. Individual risk prediction is challenging and risks of both recurrent thrombosis and bleeding differ substantially between individuals. The effects of anticoagulant prophylaxis compared to placebo in patients with high-risk of VTE has been evaluated in several meta-analyses. The data on bleeding risks have been inconsistent, but because both risks continue indefinitely, the cumulative probability of either a bleeding or a thrombotic event is high The collective conclusion is that prophylaxis is effective for preventing VTE and individual assessments must determine if patients have sufficient risk to justify the risk-benefit trade-off [8].

Strong risk factors (OR >10)	Moderate risk factors (OR 2–9)
Fracture of leg	Arthroscopic knee surgery
Hip or knee replacement	Autoimmune diseases
Hospitalization for chronic heart failure	Blood transfusion
Myocardial infarction (3 months)	Central venous lines
Major trauma	Intravenous catheters and leads
Spinal cord injury	Chemotherapy
Prior VTE	Congestive heart failure or respiratory failure
Weak risk factors (OR <2)	Erythropoiesis-stimulating agents
Bed rest >3 days	Hormone replacement therapy (formulation)
Diabetes mellitus	In vitro fertilization
Arterial hypertension	Oral contraceptive therapy
Immobility due to sitting (e.g. air travel)	Post-partum period
Increasing age	Infection (specifically pneumonia, UTI, HIV)
Laparoscopic surgery	Inflammatory bowel disease
Obesity	Cancer (highest risk in metastatic disease)
Pregnancy	Paralytic stroke

***Table 1**. Transient and persistent risk factors predisposing for venous thromboembolism, adapted from 2019 ESC Guidelines for pulmonary embolism [37].*

Evaluation of known and possible risk factors for VTE is crucial in order to weigh bleeding risk against risk for first and recurrent VTE. Risk factors for VTE are classified as acquired or inherent and are also divided into transient or persistent risk factors. VTE that is ostensibly unprovoked, has historically been labelled as idiopathic VTE and can constitute as many as 25% of events [70]. These patients are presumably burdened with one or more persistent, inherent risk factor. Clinical risk factors other than thrombophilia are demonstrated in Table 1 [37].

1.3.7 Thrombophilia investigation

Most permanent risk factors for VTE are known to the patient, but patients with idiopathic VTE may have a previously undiagnosed thrombophilia. Thrombophilia is a term for inherited or acquired hemostatic disorders with an increased coagulation tendency and with a risk of thrombotic conditions. It is associated with a moderately increased risk for VTE [71, 72] and some obstetric complications [73, 74]. Testing for thrombophilia has the potential to limit first-time VTE by indicating increased need for prophylaxis with anticoagulants, and possibly also recurrent VTE by secondary prophylaxis. The routine inherited thrombophilia panel consists of the Factor V Leiden mutation (FVL), the Prothrombin G20210A mutation (*FII c.20210G>A*), deficiencies of antithrombin, protein C and protein S, lupus anticoagulant and antiphospholipid antibodies.

The clinical indications for performing thrombophilia testing are a matter of debate because broad testing in various conditions has become increasingly common. Often with little guiding effect on treatment decisions. There are also potential negative effects of testing for thrombophilia, such as anxiety in the tested subjects, that the testing panel is expensive and that patients with a mild increase in the risk for VTE can encounter insurance discrimination [75]. A Cochrane review on thrombophilia testing for prevention of recurrent venous thrombosis concluded that it is unknown whether thrombophilia testing can actually reduce recurrence of VTE after a first episode, because there have been no prospective controlled clinical trials with thrombophilia testing as the intervention and recurrent VTE as the outcome measure [76]. There are however numerous retrospective studies on thrombophilia outcome, e.g. one large case-control study that concluded that recurrence of VTE was not reduced by testing for inherited thrombophilia in a retrospective setting [77]. There are also prospective cohort studies showing that unselected testing for heritable thrombophilia does not decrease recurrence of thrombosis [78]. The conclusion of the Cochrane review was that the assessment of whether a person should be tested or not should be completely individualized and depend on how plausible it is that testing would lead to risk reduction. Many clinical guidelines have adopted a similar approach, with recommendations of testing in limited groups of patients, primarily after early unprovoked VTE and in patients who would otherwise not receive prophylaxis [75].

1.4 POTENTIAL BIOMARKERS FOR VTE AND THROMBOPHILIA

Clinical biomarkers for a hypercoagulable state are limited to a panel of specific factors for thrombophilia and D-dimer for acute thrombosis. We need biomarkers with better specificity for acute thrombosis and increased clinical value for thrombophilia.

1.4.1 Genetical biomarkers of thrombophilia

A group of biomarkers that are already being used to some extent in the investigation of thrombophilia are the prothrombotic genetic variants, primarily single nucleotide polymorphisms (SNPs). Patients often report family histories of VTE [79] and the heritability of the disease is estimated at around 50 % in family studies [80] and 35% in GWAS. However, only ~5% can be explained by the common prothrombotic variants [81].

To date there are more than 88 known prothrombotic variants [82]. The first described genetic thrombophilia was antithrombin deficiency, described by Egeberg in 1965 [83]. Subsequently thrombophilias with strong phenotypes were discovered by hypothesis driven research and sanger sequencing [84]. The field later accelerated with the introduction of

genome wide association studies (GWAS) for first [85, 86] and recurrent VTE [87], and more recently, massive parallel sequencing [88, 89]. But despite the numerous new thrombophilia variants, the panel for inherited thrombophilia has remained unchanged since 1996, when prothrombin c.20210G>A was added [90] and the clinical value of expanded genetic panels have not been thoroughly investigated.

Amidst the controversy of thrombophilia testing, researchers of inherited thrombophilia are moving in two diametrically opposed directions. On the one hand, advocates of increased genetic thrombophilia testing see unlimited possibilities in evolution toward personalized medicine and possibly even massive parallel sequencing for risk stratification of venous thrombosis [91]. On the other hand, international bodies with perspectives that are more connected to health economy, promote a more prudent view of limiting testing to a select few individuals. No clinical guideline has yet recommended analysis of any of the emerging SNPs [92]. De Haan et al. [93] made an insert in the debate regarding which genetic variants would be of use in the clinical risk prediction of VTE, when they concluded that no additional value was added to heritable thrombophilia investigation beyond analysis of the five SNPs with the highest odds ratio (OR) for first time VTE. The study sequentially added 31 risk variants with decreasing ORs and evaluated diagnostic ability by ROC analysis, where the area under the ROC-curve (ROC AUC) increased from 0.77 for only

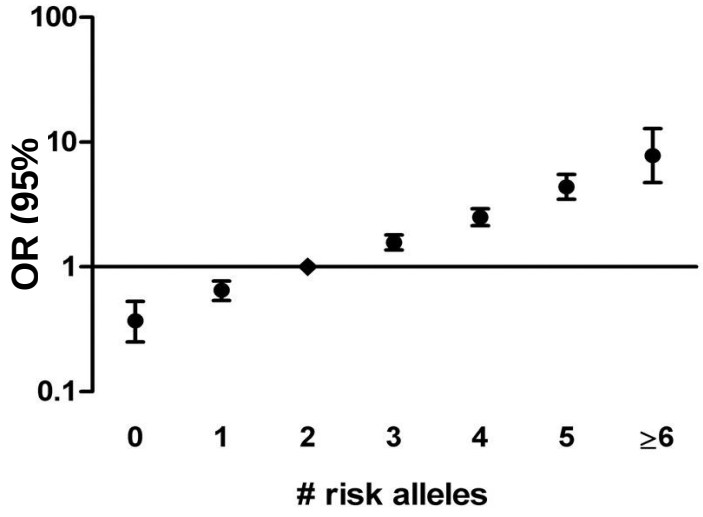


Figure 7. *Odds ratios for first venous thrombosis were calculated relative to the median number of risk alleles among control subjects, de Haan et al* [93].

clinical risk factors to 0.82 for analysis of five SNPs, with knowledge of clinical risk factors. SNPs were added in order of the OR as found in the literature, starting with FVL in the score based on only one SNP, and then adding F2, ABO, FGG and F11 in the order

presented below, and so on. OR for first-time VTE ranged from <1-1 for 0-2 risk alleles up to OR 7.48 (95 % CI, 4.49 - 12.46) for ≥ 6 risk alleles, Figure 7.

1. **Factor V Leiden c.1691G>A (rs6025)** inhibits the proteolytic inactivation of Factor V by active protein C (APC), i.e. APC-resistance [94]. It also impairs the slow activation of Factor V to the ACP-cofactor Factor V anticoagulant, resulting in inefficient FVIIIa inactivation [95]. The SNP is common in caucasians, where the prevalence is 2-10% [95]. The literature average OR for VTE is 3.79 in heterozygotes [93].

2. **Prothrombin, FII c.20210G>A (rs1799963**) located at the 3' end of the *F2* gene [96] is associated with increased but very variable prothrombin activity levels [97] The variant is present only in caucasians, with a frequency of ~2% [90].

3. **Non-O blood group (rs8176719)** is the most common genetic risk factor for VTE, with an OR of 1.85 [93]. The thrombotic risk is especially increased for pregnancy-related VTE and in women under treatment with combined oral contraceptive [98]. The thrombotic mechanism is related to the blood group structures A, B, and H(O) in the oligosaccharides of von Willebrand factor (vWF). The extensive glycosylation of vWF in blood group A and B leads to longer half-life and higher concentrations of vWF and Factor VIII in plasma [99]. In addition, ABO status has a synergistic role with other thrombophilia genetic risk factors such as Factor V Leiden and the FII c.20210G>A mutation [100].

4. **Fibrinogen c.10034C>T mutation (rs2066865)** leads to alternative splicing of the fibrinogen γ-chain, thereby reducing plasma fibrinogen γ'. Low levels of γ' is associated with an increased risk of thrombosis, because fibrin containing γ' binds thrombin more effectively ("Antithrombin 1") [101]. The SNP increases the risk of VTE in Caucasians where the prevalence is close to 25%, and the literature average OR for VTE is 1.56 [93].

5. **Factor XI, c.56-282T>C (rs2036914)** a variant in intron 2 of *F11* is common but only a mild thrombophilia factor, with OR 1.32 [93]. The increased risk of VTE is most probably caused by increased plasma levels of Factor XI. The SNP remains associated with DVT after adjustment for FXI levels, but this may be because of a closer association with average intra-individual FXI levels than with one single sample. [102].

Summation of risk alleles in the five SNPs were proposed for prediction of both first [93] and recurrent VTE [87, 103]. Other genetic panels for hereditary thrombophilia have been proposed by other groups. Soria et al. defined a clinical genetic risk algorithm for VTE risk assessment called ThromboInCode® (TiC), comprising a genetic risk score (GRS) of

twelve prothrombotic variants combined with clinical risk factors of VTE. The scores predictive capability for VTE was compared with Factor V Leiden and FII c.20210G>A, and TiC showed a discrete increase in AUC (0.68 versus 0.58, p<0.001). The addition of the FXI and FGG variants from the de Haan study did not improve the risk assessment compared to analysis by only TiC [104]. The variants in TiC are a combination of low-frequency variants with high ORs for VTE and globally common variants with low ORs. The variants included in the TiC GRS are:

1. **Factor V Leiden, FV c.1691G>A mutation (rs6025)** described above.

2. **FV Cambridge, FV c.1091G>C (rs118203905)** gives mild APC-resistance [95].

3. **FV Hong-Kong, FV c.1090A>G (rs118203906)** the A allele is associated with mild APC-resistance, although not unequivocally [95].

4. **Prothrombin, FII c.20210G>A (rs1799963)** described above.

5-8. **ABO haplotype A1, *ABO* (rs8176719, rs7853989, rs8176743 and rs8176750)** are variants in the ABO gene that predispose for haplotype A1. While group O is associated with the lowest VTE-risk, A1 is associated with the highest risk and an incidence rate ratio of 1.88 – 2.61 [98].

9. **Factor XII, FXII c.46C>T (rs1801020)**, a variant identified through a GWAS, giving increased plasma concentrations of Factor XII and susceptibility to thrombotic events [105]. Confirmed in case-control association studies as a risk factor for venous thrombosis and a thrombosis risk factor during first pregnancy. The OR of thromboembolic events is 5 times higher in homozygotic carriers than in non-carriers.

10. **Factor XIII, FXIII c.226G>T (rs5985)**, a variant with a protective effect against the risk of thromboembolism (39).The stabilization of the molecules of fibrin by the activated Factor XIII is an essential process for the formation of the clot and the V34L amino acid exchange in F XIII leads to changes in the fibrin clot structure (38).

11. **AT Cambridge II, *SERPINC1* c.36232A>G (rs121909548)** a variant causing antithrombin deficiency, type II with a decreased function in the heparin-binding domain. The variant is common and has classically been regarded as a very mild thrombotic risk factor [106], a stance which has been challenged [107].

12. Protein Z-dependent protease inhibitor, *SERPINA10* c.7928C>T (rs2232698)

The ZPI (Protein Z-dependent protease inhibitor) is a serpin inhibiting Factor Xa in the presence of protein Z, calcium and phospholipid and the Factor XIa without cofactor. Decreased levels of ZPI seem to be a mild risk factor for VTE, but some studies have reported a synergistic prothrombotic effect when combined with additional variants such as the FVL or FII c.20210G>A [108]. The nonsense variant (R67X) in ZPI that is included in TiC is associated with decreased levels of ZPI [109], but has not decisively confirmed as a risk factor for VTE [110].

A weakness of pre-assembled GRS is that the magnitude and direction of allelic effects can differ between populations. Epidemiological and genetic studies have also elucidated that there are significant ethnical differences regarding genetic etiology and incidence of VTE across populations that should also be addressed [111].Not all of the variants have been thoroughly evaluated, and based on current knowledge it is uncertain if they can be recommended for clinical thrombophilia testing [112].

Prothrombotic variants in non-coding DNA regions are very rare [90] as are synonymous variants (synonymous single nucleotide polymorphism, sSNP). Synonymous variants have long been considered silent and phenotypically neutral, since they do not impact the amino acid sequence of the protein. But several studies during the last decade have demonstrated contribution of sSNPs to disease pathology through epigenetic changes [113] and a few prothrombotic sSNPs have been reported with change in protein expression as the main mechanism [114]. Synonymous polymorphisms can in fact influence gene function in several ways including mRNA folding and stability [115]. Increased knowledge of epigenetic mechanisms will hopefully enable a better understanding of sSNP disease mechanisms. Prothrombin Belgrade (*FII* c.1824C>T, rs3136532) is one such novel sSNP, which was described in a pilot study as a potential risk factor for recurrent pregnancy loss [116] and possibly VTE.

1.4.2 Thrombin generation assays

Other biomarkers that could be of use in both thrombophilia and acute VTE are the global hemostatic assay. In thrombophilia, the global hemostatic assays can potentially be used as screening tests. Conventional screening tests such as aPTT and PT (INR) are only useful in bleeding investigations, so routine thrombophilia screening is limited to analyzing a panel of specific risk factors. These factors can only explain up to 50% of unprovoked VTE [76], indicatin that other causes of hypercoagulability likely contribute to clinical idiopathic

VTE. A dependable screening test for the hypercoagulable state could revolutionize risk prediction, since an exhaustive exploration of every coagulation component would be both unfeasible and lack the global perspective on hemostasis. Global hemostatic assays may also be in better agreement with the hypercoagulable phenotype of patients. Still, the global hemostatic assays have had a hard time establishing their clinical value as screening tests.

The global hemostatic assays are a class of assays that examine the combined effect of pro- and anticoagulant processes in plasma, because they register the complete coagulation process. Screening assays based on coagulation time, such as aPTT and PT (INR) are halted when only 3–5% of the total thrombin is formed [117, 118]. The vast majority of the thrombin production with subsequent coagulation reactions, can therefore not be observed. Hemker et al. [119] introduced the first global hemostatic assay in 1993, when they continuously measured the generation of thrombin in plasma for a prolonged time. The assay was developed into the Calibrated Automated Thrombogram (CAT), Thrombinoscope (Maastricht, the Netherlands).

Thrombin generation assays (TGA) have since shown elevated thrombin generation in intermediate and serious thrombophilic phenotypes [120, 121]. Elevated thrombin generation has also been associated with an increased risk of VTE in several studies [122-124] and unprovoked recurrent VTE in some studies [125], however not in all studies [126]. Some studies have demonstrated that thrombin generation measured in the presence of thrombomodulin allows for better appreciation of risk of recurrent VTE compared to measurement performed in the absence of thrombomodulin [127].

A few studies have evaluated the use of TGA as a complement to D-dimer in the exclusion of venous thromboembolism [128, 129], these show promising results of increased specificity combined with solid sensitivity. Theoretically, the use of TGA in acute settings may however be prone to acute phase effects which have not been extensively evaluated yet.

Of note is that the TGA still have poor interlaboratory reproducibility, due in part to non-standardized calibrators, origin of TF and phospholipids, differences in substrates, and equipment [130, 131] and sensitivity to preanalytical conditions [132]. External quality control programs are available and different standardization efforts have been made [133]. Platelet poor plasma is recommended over platelet rich plasma in automated assays, because it requires addition of tissue factor (TF) and phospholipids in defined concentrations, which facilitates standardization [132]. Normalization of results by external reference plasma has also been proposed [131, 134]. In assays with low concentrations of triggering TF (≤ 1pM), corn

trypsin inhibitor is needed to reduce random effects of contact activation [132, 133, 135]. However, the need to add corn trypsin inhibitor is somewhat controversial, mainly because of cost and because addition before blood sampling may be impractical. Despite standardization efforts reproducibility is a prevailing issue [133, 134].

There are several automated and semi-automated assays [133], measuring thrombin generation continuously using fluorogenic or chromogenic thrombin substrates. The use of a chromogenic substrate necessitates use of platelet poor plasma without fibrin [119]. The Innovance Endogenous Thrombin Potential (ETP) is a chromogenic TGA, automated on the BCS instrument from Siemens Healthcare (Erlangen, Germany) [136, 137]. The ETP uses citrated platelet poor plasma with addition of recombinant TF in a high concentration, $CaCl_2$, a non-defined fibrin aggregation inhibitor, a stabilizing buffer and the slow reacting chromogenic substrate H-β-Ala-Gly-Arg-paranitroaniline. Absorbance is continuously registered spectroscopically at 405 nm. The assay is calibrated using a proprietary calibration standard. The fact that the ETP is performed in platelet poor plasma and after the addition of a fibrin aggregation inhibitor is important for standardization, but also limits the global nature of the assay. High TF is also a key component to the reliable performance and acceptable variability in the ETP assay, but restricts thrombin generation to the extrinsic coagulation [137].

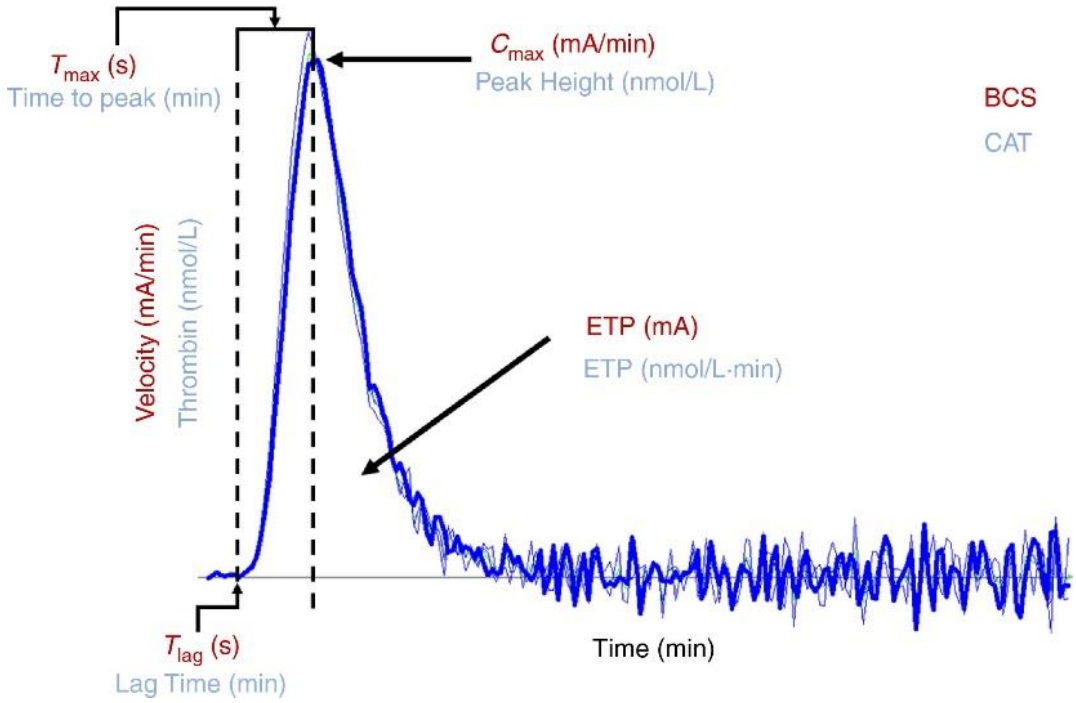


Figure 8. *Thrombogram of the Innovance ETP assay (BCS instrument) with parameters in red and the CAT assay with parameters in light blue. Illustrated reaction curve outlining the major thrombin generation parameters, Kintigh et al.*[130].

The thrombin generation parameters are calculated from the thrombin generation curve: the endogenous thrombin potential [ETP AUC], which is area under the curve, peak thrombin concentration [C_{Max}], time required to reach C_{max} [T_{Max}] and the time to start of thrombin generation [T_{Lag}], Figure 8 and Table 4.

The ETP parameter has been systematically validated as a hemostatic screening assay, reflecting both risk of thrombosis and bleeding. Specific thrombophilias have been shown to increase the ETP, with deficiency of protein S and protein C or factor V Leiden leading to an increased ETP of about 10% [120, 138, 139]. ETP can also be used to monitor bleeding tendency, and an ETP below 20% of normal predicts an increased risk of bleeding [28].

An issue that impacts the accuracy of the TGA is the existence of α-2-macroglobulin (α2M)-thrombin complexes in plasma. Thrombin inhibited by this unspecific antiprotease, is still able to cleave synthetic thrombin substrates < 8 kDa in thrombin generation assays, leading to a falsely elevated thrombin generation. The ETP mathematically corrects for the presence of α2M-trombin via calculations based on the assumption of normal α2M levels. However, concentrations differ significantly, related to age and conditions such as hepatitis C, pancreatitis or acute ischemic heart disease [130], introducing an interindividual variation in the physiological relevance of the TGA related to how extreme the α2M levels are.

1.4.3 Overall Hemostasis Potential

A theoretical drawback of the TGAs is that they do not measure the final step of the coagulation process, i.e. fibrin formation and susceptibility to fibrinolysis. However, the Overall Hemostasis Potential (OHP) provides additional information concerning the rate of fibrin aggregation and fibrinolysis. The OHP is a global hemostatic assay based on repeated turbidimetric determination of fibrin aggregation after the activation of clotting [140]. The assay originally measured the coagulation phase but has been modified to also reflect the fibrinolytic phase with the addition of tissue-type plasminogen activator (tPA). This means it reflects the integrated effect of procoagulant, anticoagulant, and fibrinolytic factors. The fibrin aggregation and lysis time parameters can also be used to calculate a proxy to fibrin clot liquid permeability [141, 142]. A similar turbidimetric global fibrin assay is the clot formation and lysis (CloFAL) assay [143].

The OHP assay has not been widely studied in thrombosis but can potentially be used as a screening assay of both hypo- and hypercoagulable conditions [144-146]. In smaller studies global fibrin generation assays have been shown to characterize the hypercoagulability after

a previous VTE [147], acute coronary syndrome [148, 149], acute stroke [150], antiphospholipid syndrome [151], diabetes [152], normal pregnancy [143], orthopedic trauma [153] and obstructive sleep apnea [154] as well as in various hypocoagulable states and anticoagulant treatment [155, 156]. In women with a history of pregnancy-provoked VTE, OHP showed hypercoagulability that could be attributed to increased fibrin generation and reduced fibrinolysis [157]. Prolonged CLT have been associated with a mild increase in of first, but not recurrent DVT [158, 159]. Prolonged CLT has also been described in patients with acute ischemic or hemorrhagic stroke, along with decreased clot permeability and increased maximum clot turbidity [160].

The assay uses a spectrophotometer (ELISA plate reader) to repeatedly register turbidity by absorbance at 405 nm, which reflects the amount of aggregated fibrin in the sample at each timepoint. In the short OHP protocol (2001), absorbance is measured every minute for 40 min [161]. The shorter protocol was optimized for use in clinical care and can sometimes be terminated before hypercoagulable samples have finished the reaction. In the longer protocol (2019), absorbance is measured every 12 seconds for 60 minutes. The longer protocol is less suited for acute settings but enables the calculation of parameters of clot formation and lysis times as a proxy for fibrin clot permeability

Coagulation is activated by addition of $CaCl_2$ and minute amounts of exogenous thrombin to platelet poor citrated plasma. In a second well on the microtiter plate, small amounts of tPA is *also* added to initiate fibrinolysis in parallel with coagulation. Two separate fibrin aggregation curves are therefore generated; the overall coagulation potential (OCP) and the overall hemostasis potential, OHP, Figure 9.

The fibrinolytic system is assessed by the overall fibrinolysis potential (OFP); the difference between the OCP and OHP areas as well as the clot lysis time (CLT), i.e. the time from 50% of maximal clotting to 50% lysis [162]. The parameters of the OHP and ETP are presented in Table 4.

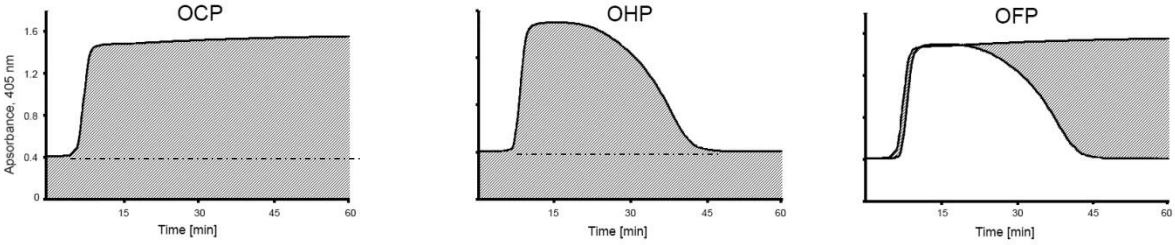


Figure 9. *Graphical presentation of the fibrin aggregation curves and the OHP parameters [163], Figure used with the kind permission of Iva Pruner.*

ETP	AUC	Area under the curve (%): Corresponds to the total amount of generated thrombin.
	C_{max}	Peak height (%): Corresponds to the maximal simultaneous thrombin generation.
	T_{lag}	Lag time (sec): Time from the start of measurement until the initiation of thrombin generation.
	T_{max}	Time to peak (sec): Time from the start of measurement until the time of maximal thrombin generation.
OHP	OCP	Overall Coagulation Potential (abs sum): Area under the fibrin generation curve, without fibrinolysis.
	OHP	Overall Hemostatic Potential: Area under the fibrin generation curve expressed by a summation of the absorbance values, with fibrinolysis (addition of tPA).
	OFP	Overall Fibinolysis Potential (%): The difference between the OCP – OHP curves. Determined by the fibrinolytic system and the density and thickness of the fibres; thin fibres give a denser fibrin network and hence a slower degradation rate [164].
Clot turbidity assay	Lag time	time point when exponential growth of the absorbance occurs
	Max abs	the median absorbance value of three consecutive points where the curve reached a plateau less the lag turbidity
	Clotting rate	the slope of line fitted from the point at the start of the exponential growth curve to the point reaching the plateau
	Max abs time	the time to the plateau (without t-PA) or peak (with t-PA)
	Slope time	the duration of the exponential growth curve
	CLT	clot lysis time; time from Max abs time and return to baseline

Table 4. *Parameters of the ETP and OHP, clot turbidity calculated from the OHP.*

1.4.4 Additional markers of hypercoagulability

The aspects of coagulation which is reflected neither in the thrombin nor the fibrin generation assay, are the procoagulant and fibrinolytic activities related to blood flow characteristics and cellular elements. Namely because vascular components, hematologic cells and to varying extent also microvesicles are absent in plasma. Whole blood global hemostatic methods such as thromboelastography could potentially be one step closer to the in vivo coagulation, although these whole blood assays have very poor reproducibility and must be performed on fresh samples within a few hours of sampling [165].

Another alternative, to capture at least one feature of the cellular aspect of hemostasis is measurement of cell derived microvesicles, which has the advantage of being possible in plasma. One additional aspect that is not reflected in any global hemostatic assay is the fibrin clot structure which can be assessed by liquid permeability, light scattering (turbidimetry), confocal microscopy and scanning electron microscopy. The visualization of fibrin clot networks allows for measurement of such variables as the fiber thickness and length, network density and porosity and the number of branch points.

2 AIMS

The aim of this thesis was to improve laboratory diagnostics of venous thromboembolism and thrombophilia in close collaboration between research and clinical laboratories.
The specific objectives of the studies were:

Study I

- Verify that age-adjusted D-dimer cut-offs for suspect acute venous thromboembolism (VTE) increased the specificity and reduced the rate of false-positive D-dimer results for older patients, without compromising the sensitivity
- Compare four D-dimer assays common in Sweden, in order to facilitate the nation-wide implementation of age-adjusted D-dimer cut-offs for VTE

Study II

- Compare the discriminatory accuracy of global plasma-based hemostatic assays, fibrin monomers and D-dimer, in the assessment of suspected acute VTE

Study III

- Assess the prevalence of hypercoagulable profiles in patients with a clinical indication for thrombophilia investigation, using plasma based global hemostatic assays
- Assess the prevalence of hypercoagulable profiles in patients with conventionally verifiable thrombophilia

Study IV

- Investigate the frequency of the Factor II Belgrade variant in a Serbian population of patients with previous venous or arterial thromboembolism
- Functionally characterize Factor II Belgrade to substantiate if the synonymous variant is associated with an increased thrombotic risk

Study V

- Validate the applicability of a Spanish genetic risk score (TiC) in Swedish patients
- Evaluate the discriminatory accuracy of a new genetic risk score compared to current genetic analysis in patients investigated for thrombophilia

3 METHODS

3.1 STUDY POPULATION AND STUDY DESIGN

3.1.1 Acute Venous Thromboembolism (Study I and II)

Study I and II formed a prospective case-control study investigating new approaches to laboratory diagnosis of suspect acute pulmonary embolism or deep venous thrombosis in a lower limb. Between April 2014 and May 2015, 954 consecutive out-patients were recruited from the emergency department of Karolinska University Hospital in Huddinge. The patients were included regardless of clinical probability of VTE and were sampled at the emergency department before initiation of any treatment. Study I evaluated age adjusted cutoffs in four D-dimer assays in 940 patients remaining after exclusions. Study II compared plasma-based global hemostatic assays to FM and D-dimer in a subset of 158 patients, which corresponded to all available patients with VTE (n = 60) and patients without VTE as controls (n = 98), randomly selected after age- and sex-stratification (Figure 10). Clinical data was retrospectively collected from patients' medical records, blinded for assay results.

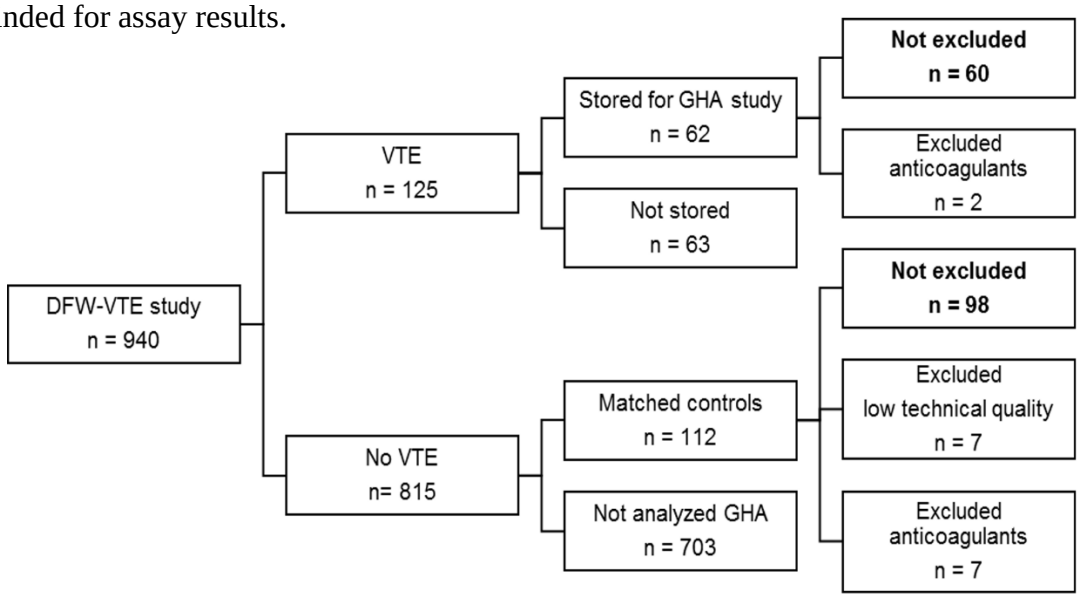


Figure 10. *Flow chart of samples in study II.*

VTE was radiologically verified (n = 125) or, in patients without VTE, excluded radiologically (n = 391) or by a three month follow up of medical records (n = 424). Isolated thrombophlebitis was present in 9 cases and was classified as negative for VTE. Radiology was accredited according to ISO/IEC 17025 by the Swedish Board for Accreditation and Conformity Assessment and was performed on the day of sampling in 83 patients, the following day in 15 patients, three days after sampling in three patients and after seven days in two patients. Radiology consisted of CTPA or ventilation/perfusion lung

scintigraphy for PE and doppler ultrasonography for suspected DVT. In 10 patients with suspected DVT, the only radiography consisted of lower-limb vein compression ultrasonography (CUS), performed at the emergency department

3.1.2 Thrombophilia (Study III and V)

Study III and V compounded the KUL thrombophilia study, a cross-sectional descriptive study. All adult patients that were subject to thrombophilia investigation at the special coagulation lab between 2014-2016 were invited to participate. Inclusion criteria were chosen to represent patients with a clinically robust indication for thrombophilia testing. After exclusions there were 369 patients, which were 166 with the inclusion criteria of personal history of VTE < 50 years of age, 153 with at least one first grade relative with VTE < 50 years of age and 50 with both of those inclusion criteria. A flow chart of the plasma samples that were included in study III is presented in Figure 11. Medical history was obtained from standardized forms filled out by the patient at the time of the written consent. The forms included gender, age, smoking, diabetes, pregnancy, BMI, family history of VTE and use of prothrombotic hormonal contraceptives or anticoagulant medication. Additional data and data verification were retrospectively assembled from electronical medical records, including details of any VTE, detailed family history of VTE, acquired risk factors for VTE, medication and results of thrombophilia investigation.

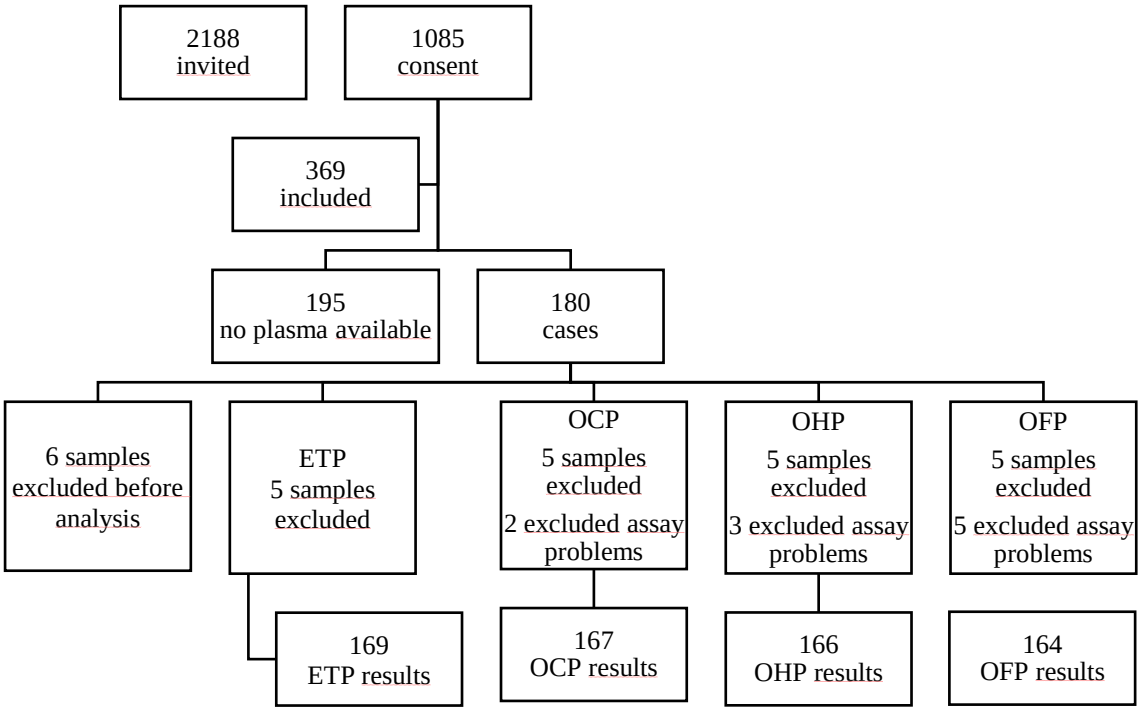


Figure 11. *Flow chart of samples in study III*

Study III was an evaluation of global hemostatic assays in patients investigated for thrombophilia. Result of the thrombophilia investigation were collected from the laboratory information system. The samples were additionally analyzed for the previously described variants in *FGG, F11* and *ABO* (non-O), Table 3.

Verifiable conventional thrombophilia was divided into; moderate thrombotic risk: protein C < 0.6 kIU/L, protein S < 0.5 kIU/L, antithrombin < 0.7 kIU/L, lupus anticoagulant >1.4 in normalized dRVVT- or aPTT-quote or homozygous FVL or FII c.20210G>A . Mild thrombotic risk: heterozygous FVL and/or FII c.20210G>A [90]. In the expanded genetic panel (*FVL, F2, FGG, F11* and *ABO*), thrombophilia was defined as ≥ 3 risk alleles [93].

Study V was an evaluation of a commercial clinical genetical risk algorithm (CGRA), TiC [104]. The CGRA includes 12 variants (Table 2) together with the clinical parameters; age, gender, BMI, smoking, diabetes and family history of VTE. In women, it incorporates pregnancy and treatment with prothrombotic hormonal contraceptives. A global risk value is calculated with the use of risk coefficients assigned to each risk factor in the algorithm, with adjustment for certain combinations. The risk coefficients are based on published information and meta-analyses (supplemental material of [104]). TiC was also compared to purely genetic risk scores (GRS) and other CGRA.

Variant	**SNP**	**TiC risk coefficient**	**OR**
*F5 (Leiden, G/**A**)*	rs6025	1.589 (htz)	3.79
*F2 (20210 G/**A**)*	rs1799963	0.293 (htz)	2.78
*ABO non-O (*G/**del**)	rs8176719		1.85
*FGG (*C/**T**)	rs2066865	0.344 (hmz)	1.56
*F11 (**C**/T)*	rs2036914	0.293 (htz)	1.32
*F5 (Hong Kong **A**/G)*	rs118203905	1.589 (htz)	-
*F5 (Cambridge G/**C**)*	rs118203906	1.589 (htz)	-
*F12 (C/**T**)*	rs1801020	1.633 (hmz)	5.12
*F13 (**G**/T)*	rs5985	0.198 (hmz)	0.82
*AT (Cambridge II G/**T**)*	rs121909548	2.277 (htz)	1.20
*Prot Z dependent inh (C/**T**)*	rs2232698	1.358 (htz)	3.98
ABO A1	rs7853989 rs8176743 rs8176750	0.956	1.88 – 2.61

Table 2. *Comprehensive summary of analyzed SNPs, adapted from Hinds et al [86], Vasan et al [98], Soria et al. [104] and Croles et al [106]. Risk alleles in bold letters.*

3.1.3 Mixed venous and arterial thrombosis cohort (Study IV)

Study IV was an investigation of a novel prothrombotic genetic variant, FII c.1824C>T (rs3136532). The study was planned to also include a confirmation study in the KUL thrombophilia cohort, but the confirmation study is instead being performed separately and is not included in this thesis. Hence, the cohort in study IV is the Belgrade cohort which was assembled by I. Pruner. The 489 cases were included from a database of 4500 patients referred for routine thrombophilia screening (2002–2016), Figure 12. Inclusion criteria were personal history objectively documented DVT, pulmonary embolism or cerebrovascular insult. Cases and healthy controls were genotyped for FII c.1824C>T . Phenotype characterization was then performed in six cases with heterozygous FII c.1824C>T , six with heterozygous FII c.20210G>A and 11 controls without FII c.20210G>A or FII c.1824C>T . Characterization consisted of routine coagulation assays, prothrombin levels, global hemostatic assays and fibrin clot characterization.

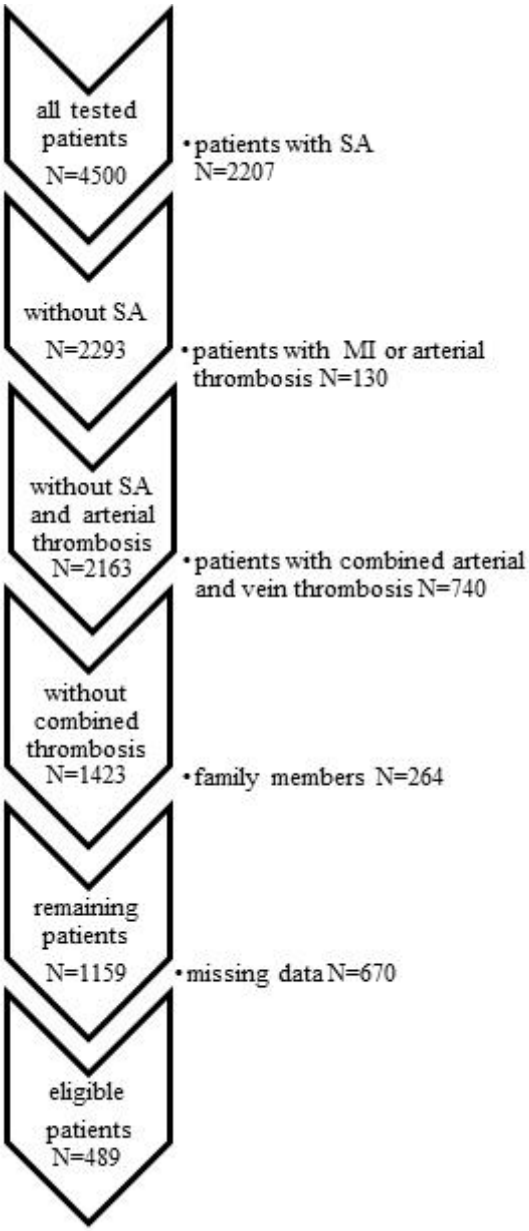


Figure 12. Flow chart of samples in study IV.

3.1.4 Healthy controls

Healthy medical students were enrolled for analysis of ETP (n = 38), OHP (n = 42) and fibrinogen (n = 42). The controls were used to verify the observation that both cases and controls included from the emergency department displayed hypercoagulable profiles. They were also used to calculate 95th percentile ranges for the 2019 modification of the OHP for study III [166]. Healthy blood donors were also collected for study IV and V. For study IV, 432 Serbian donors, with no thrombotic disorder, family history or known thrombophilia. For study V, 100 Swedish donors, with no thrombotic disorder, family history or anticoagulant medication, age- and sex-matched to the KUL thrombophilia cohort.

3.2 PREANALYTICAL PROCEDURES

D-dimer, FM and PT (INR) were analyzed in fresh platelet poor plasma (PPP) (0.109 mol/L (3.2%) Na-Citrate), whereas ETP, OHP, fibrinogen, PT(INR), aPTT, antithrombin were analyzed after storage of the same samples at -70^{o} C and thawing in a 37° C water-bath. Plasma for the thrombin generation assay was not separately collected and, accordingly, no Corn Trypsin Inhibitor was added at sampling [133], as per the proprietary instructions for the ETP [130, 136].

DNA was extracted from leukocytes in EDTA blood by digestion and selective precipitation with ethanol on an automated QiaCube system using the QIAamp DNA-blood mini kit (Qiagen, Düsseldorf, Germany) and stored at -20°C.

Sample aliquotation and storage for study II was performed within one hour after analysis, in the acute clinical chemistry lab in parallel with routine operations. More than 2200 aliquots were stored and a subset of these were used for study II. Because sample collection was performed in a routine clinical chemistry laboratory where storage of research samples could not be the main priority, only a subset of samples from study I could be saved for analysis by FM in fresh plasma and stored for study II. In study II we included all samples with VTE and randomly selected age- and sex-matched samples without VTE. All included samples that were available (that had been stored) were analyzed by ETP and OHP (n=174; 62 VTE and 112 without VTE). After exclusions 158 samples remained.

Samples for the KUL thrombophilia cohort were initially collected and handled in the routine preanalytical pathway of the Karolinska University Laboratory, accredited according to ISO15189 by the Swedish Board for Accreditation and Conformity Assessment. After written informed consent was obtained, the 250 patients who fulfilled the inclusion criteria were stored in the study biobank if their blood samples were not yet discarded (i.e. within two months of arrival to the clinical lab).

Samples for study V were collected specifically for the study. All subjects were without anticoagulant therapy and without thrombotic events within 6 months prior to sampling.

3.3 ASSAYS

D-Dimer, FM and PT (INR) were analyzed on a Sysmex CS-2100i (Siemens Healthcare Diagnostics, Erlangen, Germany), except Stagos STA®-Liatest® D-Di Plus which was analyzed on the Stago CompactMax (Diagnostica Stago, Asnières-sur-Seine, France). ETP, Protein C, protein S, lupus anticoagulant and Factor II activity was analyzed on the BCS® XP instrument (Siemens). Fibrinogen, antithrombin and aPTT were analyzed on the Sysmex CS-5100 (Siemens) and CRP was analyzed on the Cobas 6000 instrument (Roche Diagnostics, Basel, Switzerland). All assays were calibrated with proprietary calibrators.

3.3.1 Fibrin degradation products (Study I-III)

D-dimer and FM were analyzed by rapid particle-enhanced immunoturbidimetric assays; Roche Tina-quant D-dimer (Roche Diagnostics), Siemens INNOVANCE® D-dimer (Siemens), Medirox MRX D-dimer (MRX143) (Medirox, Nyköping, Sweden) and STA®-Liatest® D-Di Plus and STA®-Liatest® FM (Diagnostica Stago). Cutoffs and number of samples tested are presented in Table 3.

	Samples	Cutoff	Age adjusted cutoff
Tina-quant D-Di	939	<0.50 mg/L FEU	Age x 0.01
Innovance D-Di	871	<0.50 mg/L FEU	Age x 0.01
MRX D-Di	819	<0.20 mg/L	Age x 0.004
STA-Liatest D-Di	170	<0.50 mg/L FEU	Age x 0.01
STA-Liatest FM	119	<6 mg/L	-

Table 3. *Details of D-dimer assay cutoffs.*

3.3.2 Routine coagulation and chemistry assays (Study I - IV)

PT (INR) was analyzed by MRX Owrens PT (Medirox). Fibrinogen was analyzed using the Dade® Thrombin Reagent (Siemens) which is a modified Clauss assay. Antithrombin was analyzed with the Factor II-based Berichrom® Antithrombin III (Siemens) in study II and IV and by the Factor Xa-based Innovance® Antithrombin assay (Siemens) in study III. Activated partial thromboplastin time (aPTT) by PTT-Automate (Diagnostica Stago) and CRP by the immunoturbidimetric CRPL3, C-Reactive Protein Gen. 3 assay.

3.3.3 Thrombophilia assays (Study I - IV)

Protein C was analyzed using the enzymatic Berichrom® Protein C assay (Siemens), free protein S was analyzed using the Innovance Free PS Ag (Siemens) and lupus anticoagulant was analyzed according to the ISTH guidelines [167] using the following reagents: diluted

Russel viper venom time (dRVVT) by LA Screen and LA confirm (Life Diagnostics, West Chester, Pennsylvania, USA), PTT-LA (Diagnostica Stago, Asnières-sur-Seine, France) and Actin FS PTT (Siemens).

To avoid false positive thrombophilia classification, results were excluded in case of low protein C or S from subjects on warfarin medication, protein S from pregnant patients, low antithrombin from patients on direct oral F Xa-inhibitors or positive lupus anticoagulant from patients on anticoagulant, if they had not been verified after 12 weeks in a sample without medication.

3.3.4 Prothrombin in plasma (Study IV)

Prothrombin (FII) activity was determined using the Dade® Innovin® Reagent with factor II-deficient plasma (Siemens) and FII protein concentration was determined using the *Human Prothrombin ELISA kit* (Nordic BioSite, Täby, Sweden).

Relative plasma FII levels were also quantified by western blot in triplicate in three separate experiments. The samples were separated on 12% SDS–PAGE and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore®, Sigma Aldrich, St. Louis, Missouri, United States). *Thrombin K-20 goat polyclonal* was used as the primary antibody (Santa Cruz Biotechnology, Dallas, Texas, United States) and *anti-goat IgG conjugated with peroxidase* (Sigma Aldrich) as the secondary antibody. Immunoreactive bands dyed with chemiluminescent ECL blotting reagents (Millipore®). The signal was relatively quantified by densinometry in the Image Studio Lite (LI-COR Biosciences, Lincoln, Nebraska, United States) and normalized against standard human plasma.

3.3.5 Global hemostatic assays (Study II, III and IV)

Thrombin generation was analyzed using the automated Innovance® ETP assay with the proprietary B-settings for hypercoagulable patients, where thrombin generation is restricted to the extrinsic coagulation by using Tissue Factor in high concentration (300 pM) as an activator [137]. The assay includes an unnamed fibrin aggregation inhibitor and a slow reacting chromogenic thrombin substrate (H-β-Ala-Gly-Arg-pNA).

The OHP was analyzed with $CaCl_2$, 0.04 U/mL thrombin and 300 ng/mL t-PA and the two separate protocols for clinical laboratories. The 2001 protocol [161] was used in study II and the OCP, OHP and OFP in study IV. The 2019 protocol [166] was used in III and for the turbidity times in study IV. The ETP results and OHP results from study III were

normalized against standard human plasma, giving results in %. Intra-assay and inter-assay CV% for the ETP were 3.3% and 2.7% respectively. Intra-assay and inter-assay variation coefficient (CV%) for the OHP were 9.3% and 12.3%.

3.3.6 Fibrin clot characterization (Study I-IV)

Samples from study IV were analyzed by SEM to visualize the structure of fibrin networks in FII c.1824C>T . Patient plasma was mixed with $CaCl_2$ (1 mol/L) and 0.05U/mL of thrombin and transferred to plastic tubes before incubation in a humidity chamber. The clots were washed for 30 min in PBS, fixed in 2% glutaraldehyde for 60 min at room temperature and then stored at 4°C. The specimens were rinsed in distilled water and placed in 70% ethanol/10 min, 95% ethanol/10 min, absolute ethanol/15 min, pure acetone/10 min and then transferred to tetramethylsilane (Merck) for 10 min and air-dried. After drying, the specimens were mounted on an aluminum stub, coated with carbon (Bal-Tec MED 010) and analyzed in an Ultra 55 field emission scanning electron microscope (Carl Zeiss) at 3 kV. For each sample, 50 individual fibers were randomly selected for measurement of thickness using SIS iTEM software (FEI Company, Hillsboro, Oregon, United States).

Samples from the VTE and thrombophilia cohorts were prepared for analysis of fibrin networks by confocal microscopy and SEM. A subset of samples from the VTE-cohort were investigated by fibrin clot permeability, but fibrin clot characterization of these cohorts was removed from the doctoral project and will be presented separately.

3.3.7 Genotyping Prothrombotic variants (Study III, IV and V)

Genotyping of the variants FVL (rs6025), *FII c.20210G>A* (rs1799963), ABO non-O (rs8176719), FGG (rs2066865) and F11 (rs2036914) were performed by TaqMan SNP Genotyping Assays in the ABI 7500 Fast Real-Time System (Applied Biosystems, Foster City, California, United States) Genotyping of the variants included in the TiC panel were performed at the genetics lab of *Sant Pau Hospital* (Barcelona, Spain) using KASPar, Competitive Allele Specific PCR SNP genotyping system (KBiosciences, Hoddesdon, United Kingdom). Cases and controls from study IV were tested for *FII* c.1824C>T (rs3136532), FVL and FII c.20210G>A restriction fragment length polymorphism (RFLP) to determine the EAF. FII c.1824C>T was additionally genotyped by sanger sequencing.

3.3.8 Gene expression in transfected cell cultures (Study IV)

Wild-type full-length *F2* cDNA was cloned in the pCI-neoΔSV40 vector, which promotes constitutive expression of cloned DNA inserts. For stable expression of the genes, the vector was co-transfected with an expression vector and the neomycin resistance gene was employed as a reference gene. The *FII* 20210A and FII 1824T variants were introduced by site-directed mutagenesis, also resulting in stable expression. The Cos-7 cells were cultivated for 4 weeks in conditioning medium with G418 (1 mg/mL) and stored in liquid nitrogen after harvest.

The level of prothrombin mRNA was determined by reverse transcription - real time PCR (RT-qPCR), using the TaqMan allelic discrimination method in the Applied Biosystems 7500 Real-Time PCR system. Total mRNA was isolated using the Qiagen RNeasy Plus Mini Kit (Qiagen) and reverse transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Results were analyzed by software provided with the ABI Prism 7500 Sequence Detection System (Applied Biosystems). The relative mRNA concentration was compared between transfected and wild-type cells.

Effects of Prothrombin Belgrade on the mRNA secondary structure were predicted *in silico*

3.4 ETHICAL AND LEGAL ASPECTS

All studies were conducted in accordance with the Declaration of Helsinki and all participants provided a written informed consent at enrolment. Studies I-III and V were approved by the regional ethics review board in Stockholm (DNR 2013/2143-31 and 2014/987-31). Study IV was approved by the local ethics committee at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade (O-EO-004/2015/2).

The Swedish samples were registered as sample collections in the Stockholm Medical Biobank. The personal data processing databases were registered at Karolinska University Hospital. When the pseudonymized database was shared with the Spanish diagnostics company of FerrerInCode for analysis in study IV, the data was pseudonymized a second time. The transfer of data and genetic material was detailed in a Material Transfer Agreement (MTA) and a Personal Data Processors Agreement (PDPA) that was drafted between Karolinska Institutet, Stockholm Medical Biobank and FerrerInCode. All samples were discarded after analysis in Spain, as specified in the MTA and a separate attestation of sample destruction.

4 RESULTS

4.1 PATIENTS WITH SUSPECTED ACUTE VTE (STUDY I AND II)

4.1.1 Evaluation of age-adjusted D-dimer cutoffs

All D-dimer assays had AUCs ≥0.9, the diagnostic performance is displayed in Table 5 and Figure 13. The sensitivities of all assays were high, with only six cases falsely classified as negative by any assay. When age adjusted cutoffs were applied, all assays maintained their sensitivities and the specificities increased by 6-7%.

	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	NPV	% Neg	% FP
Tina-quant	0.93 (0.91-0.95)	0.98 (0.95-1.00)	0.63 (0.59-0.66)	0.99	55%	32%
D-Di	-	0.98 (0.95-1.00)	0.69 (0.66-0.72)	0.99	60%	27%
Innovance	0.92 (0.89-0.95)	0.96 (0.93-1.00)	0.61 (0.58-0.65)	0.99	53%	34%
D-Di	-	0.96 (0.93-1.00)	0.68 (0.64-0.71)	0.99	59%	28%
MediRox	0.90 (0.87-0.94)	0.94 (0.90-0.99)	0.66 (0.62-0.69)	0.99	58%	30%
D-Di	-	0.94 (0.90-0.99)	0.72 (0.69-0.76)	0.99	64%	24%
STA-Liatest	*0.89 (0.82-0.96)*	*0.96 (0.89-1.00)*	*0.51 (0.43-0.59)*	*0.99*	*43%*	*41%*
D-Di	-	*0.96 (0.89-1.00)*	*0.58 (0.50-0.66)*	*0.99*	*49%*	*35%*

Table 5. *Diagnostic performance of the four D-dimer assays, comparison of proprietary cutoffs and age-adjusted cutoffs for each assay in 954 patients.*

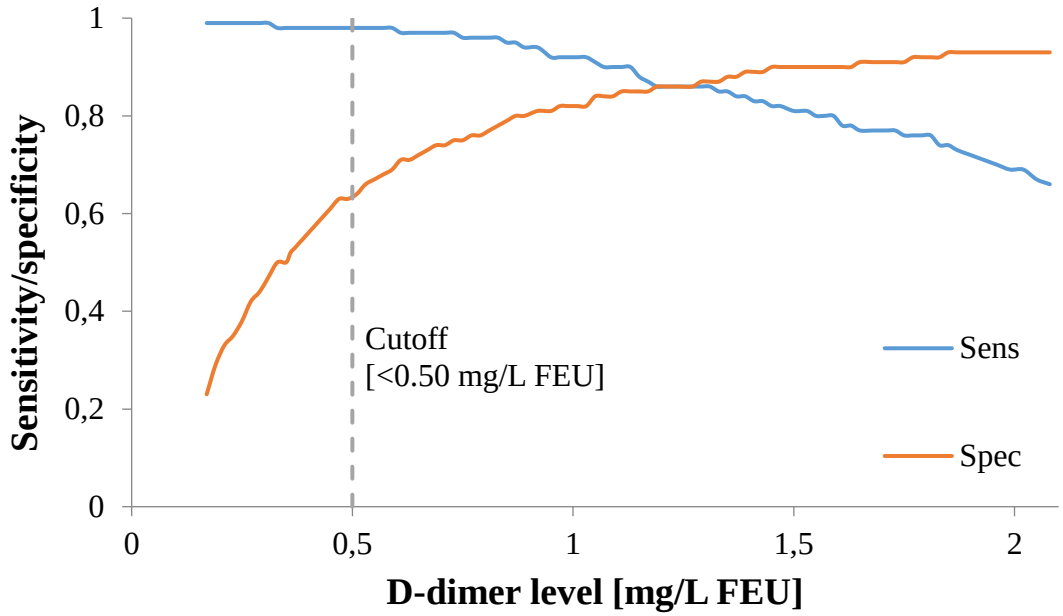


Figure 13. *Cumulative data analysis of Tina-quant D-dimer, similar curves for all assays.*

The decrease in number needed to test was also modest; 0.15-0.27 for the respective assays. The false positive results decreased by 5-6 percent units, with a corresponding increase in negative results for all assays as those false positives became true negatives. The decrease in % FP increased by age, in patients ≥ 80 years it decreased by up to 20%, see Figure 14.

In patients < 50 years, no adjustment of the proprietary cutoff was applied. D-dimer analyzed by Tina-quant displayed a sensitivity of 0.91 (0.82-1.01, 95% CI) and specificity 0.82 (0.78 – 0.86) in patients < 50 years (n = 346).

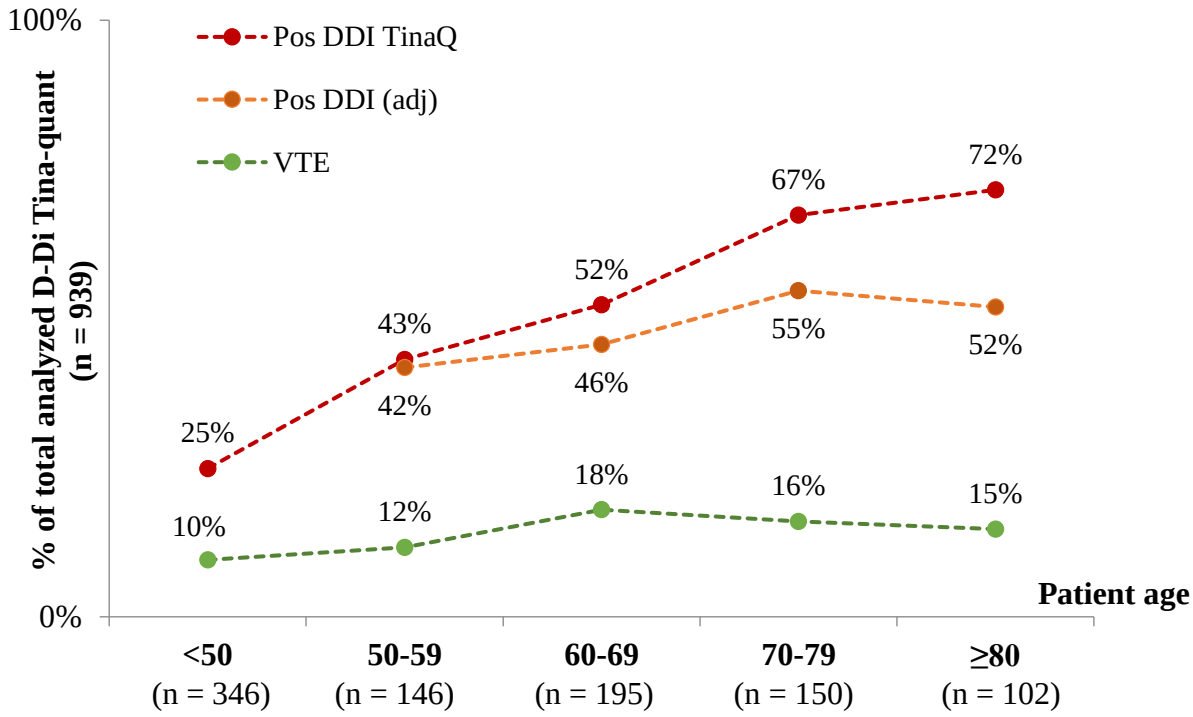
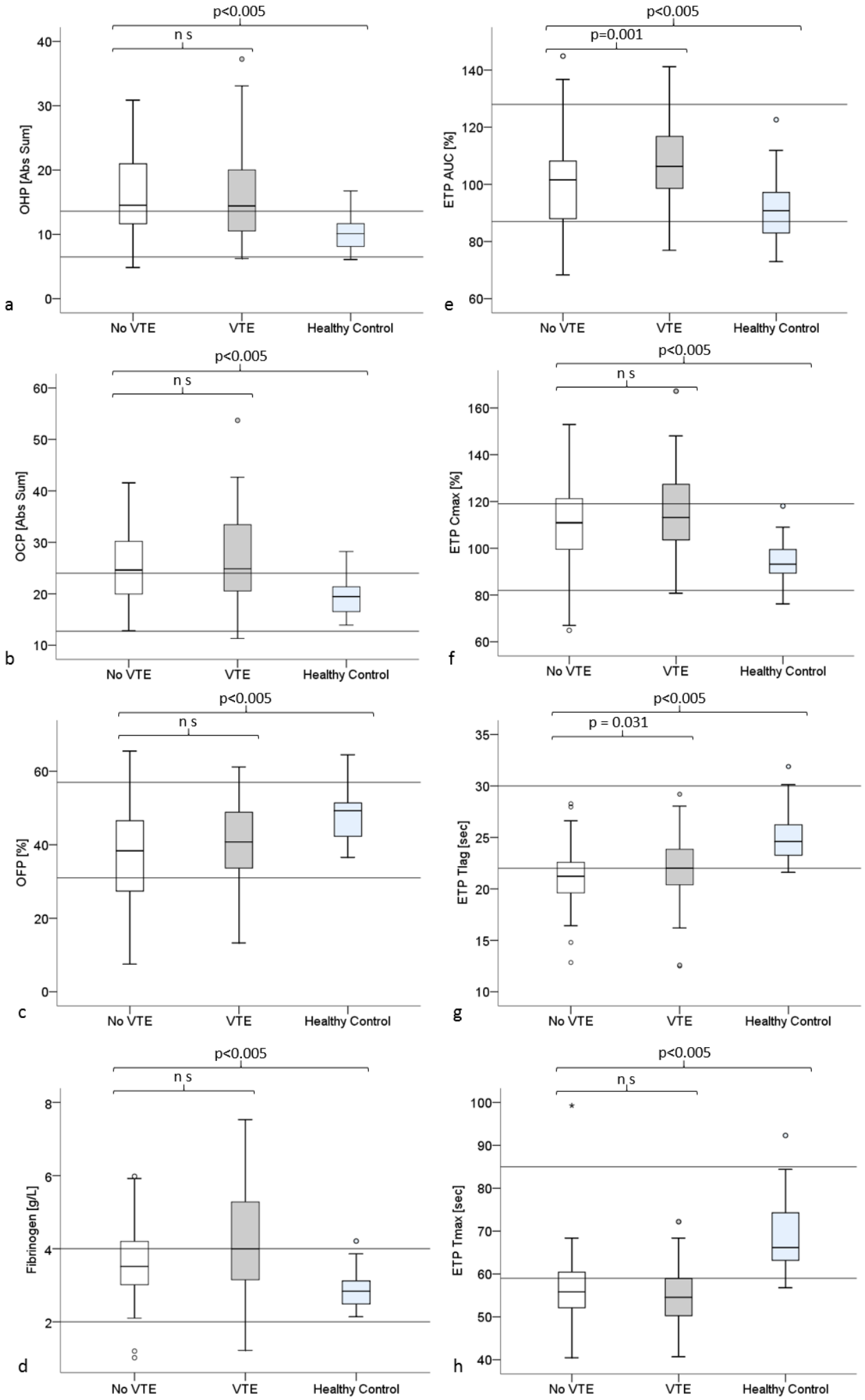


Figure 14. *Difference between proprietary and age-adjusted cutoff, presented as a percentage of the total VTE occurrence and percentage of positive results in each age group. For each age group, n is designated in parenthesis.*

4.1.2 Global hemostatic assays in patients with venous thromboembolism

All OHP and ETP parameters as well as fibrinogen demonstrated hypercoagulable profiles, with significant differences in medians compared to healthy controls, see Figure 15 . Significant differences between patients with and without VTE were found in D-dimer and FM ($p < 0.005$), ETP T_{lag} ($p = 0.031$) and for ETP AUC ($p = 0.001$), where the mean for VTE was 99% and non VTE 107%. Assessment of the relationship between ETP AUC and OHP levels in these clinical subgroups showed no trends in relation to VTE status.

Figure 15. *Global hemostatic parameters in patients with no VTE [white], VTE [grey] and healthy controls [light grey].* Reference lines at 95th percentile reference ranges.


a
OHP [Abs Sum]
p<0.005
n s
No VTE
VTE
Healthy Control
b
OCP [Abs Sum]
p<0.005
n s
No VTE
VTE
Healthy Control
c
OFP [%]
p<0.005
n s
No VTE
VTE
Healthy Control
d
Fibrinogen [g/L]
p<0.005
n s
No VTE
VTE
Healthy Control
e
ETP AUC [%]
p<0.005
p=0.001
No VTE
VTE
Healthy Control
f
ETP Cmax [%]
p<0.005
n s
No VTE
VTE
Healthy Control
g
ETP Tlag [sec]
p<0.005
p = 0.031
No VTE
VTE
Healthy Control
h
ETP Tmax [sec]
p<0.005
n s
No VTE
VTE
Healthy Control

The diagnostic performance of the parameters of the OHP and ETP assays compared to Tina-quant D-dimer and FM is presented in Figure 16 and Table 6. The parameters of OHP and ETP had ROC AUCs ≤0.65. The specificities ranged from 0.0-0.2 at the respective cutoff where the sensitivity of each parameter was in accordance with CLSI H59-A and equal to the sensitivity of D-dimer (0.97 at the cutoff 0.5 mg/L FEU).

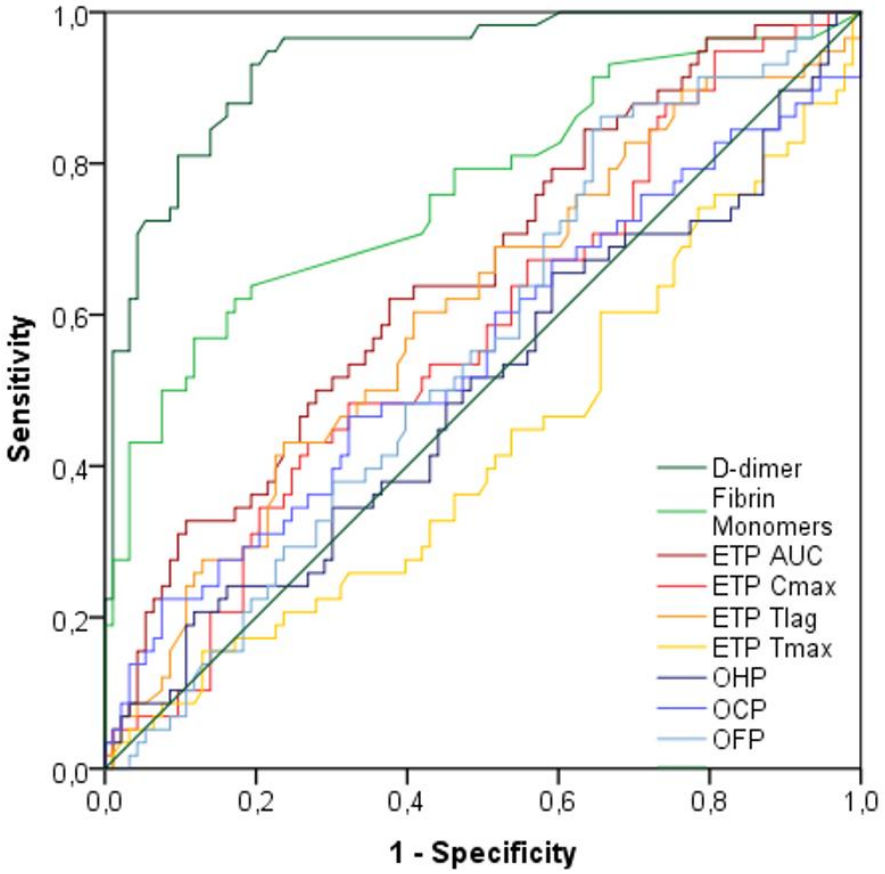


Figure 16. *Diagnostic performance in discrimination of acute VTE presented as ROC-curves for D-Di, FM, ETP and OHP. ETP T_{max} is negative because it is the only parameter where a smaller test result indicates a more "positive" test*

Parameter	ROC AUC (95% CI)	Cutoff	Sensitivity	Specificity
OHP [Abs Sum]	0.50 (0.40 - 0.60)	7	0.97	0.04
OCP [Abs Sum]	0.55 (0.45 - 0.65)	12	0.97	0.00
OFP [%]	0.56 (0.47 - 0.65)	17	0.97	0.09
ETP AUC [%]	0.65 (0.56 - 0.74)	85	0.97	0.20
ETP C_{max} [%]	0.57 (0.48 - 0.67)	90	0.97	0.13
ETP T_{lag} [sec]	0.60 (0.51 - 0.70)	15	0.97	0.02
ETP T_{max} [sec]	0.43 (0.33 - 0.52)	42	0.97	0.01
D-dimer [mg/L FEU]	0.94 (0.90 - 0.97)	0.5	0.97	0.66
Fibrin Monomers [mg/L]	0.76 (0.68 - 0.85)	0.9 6.0	0.97 0.57	0.20 0.84

Table 6. *Areas under the ROC curve and accuracy parameters at the cutoff fulfilling CLSI H59-A. For FM the proprietary cutoff is also presented.*

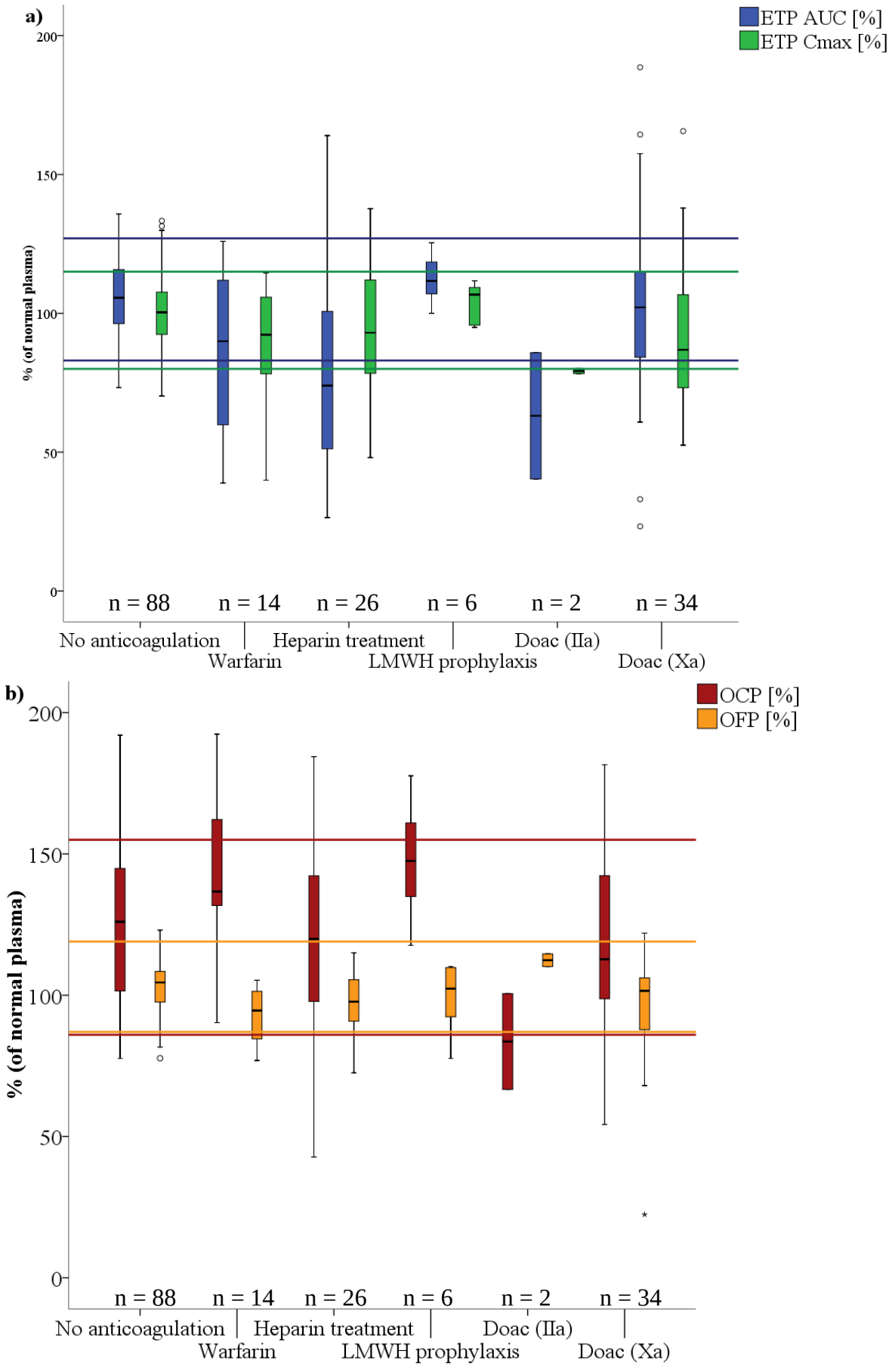


Figure 17. Assay result dispersion for a) ETP AUC and ETP C_{max} and b) OCP and OFP related to type of anticoagulant medication. Reference lines at 95th percentile reference ranges.

4.2 PATIENTS INVESTIGATED FOR THROMBOPHILIA (STUDY III AND V)

4.2.1 Global hemostatic assays in thrombophilia investigation

Plasma from 174 patients were available for statistical analysis in study III, see Figure 11. The FH group had a lower median age, compared to the other groups, with a high proportion of females and pregnancies (40% of female participants). Anticoagulant medication was present in ≥80% of patients in the VTE groups, but 6% in the FH group.

Hypercoagulability profiles are presented in Figure 17 and Table 7. There were no significant differences between the distributions of the clinical indication groups (*VTE*, *VTE+FH*, *FH*). Increased risk of venous thrombosis was demonstrated by GHA in 59 patients (34%) with 17 patients (10%) that showed increased thrombotic risk in both GHA and the conventional thrombophilia panel. Only 3 of the 14 patients with verifiable moderate-risk thrombophilia were identified by the global hemostatic assays; two patients homozygous for FVL that had increased thrombin generation and one patient with APS and decreased fibrinolysis in the OHP assay. There were no robust correlations between the global hemostatic assays and verifiable thrombophilia, although notably there were only 26 thrombophilia patients without anticoagulant medication.

The conventional thrombophilia demonstrated increased risk of VTE in 54 (31%), where 14 patients (8%) were at moderate risk and 40 patients (23%) had only a mildly increased risk of VTE. The addition of three more SNPs to the conventional panel increased the number of patients with a verifiable increased risk of thrombosis, n = 67. The extended thrombophilia panel demonstrated 90 patients (52%) with 3-4 risk alleles, 21 (12%) with 5-6 risk alleles and 10 (6%) with biochemical markers of thrombophilia.

In total, 84 patients had anticoagulant medication at the time of sampling and 90 did not. Preliminary results show that the median number of days since last VTE was 21 (IQR 4 – 156 days). We tried to objectify the extent of samples taken in suboptimal preanalytical conditions by quantifying the proportion of common preanalytical pitfalls. We found that lupus anticoagulant had been ordered in 82% of samples receiving anticoagulant medication, antithrombin analyzed by FXa-method had been ordered in 67% of patients receiving Factor Xa-inhibiting DOACs, protein C had been ordered in 86% of patients receiving warfarin and that protein S had been ordered in 62% of pregnant patients.

		Total *(n = 174)*	VTE *(n = 83)*	VTE + FH *(n = 19)*	FH *(n = 72)*
***Global hemostatic assays**, n positive (p)*					
ETP AUC	*(n = 169)*	16 (0.09)	8 (0.10)	3 (0.16)	5 (0.07)
ETP C_{max}	*(n = 169)*	22 (0.13)	11 (0.14)	3 (0.16)	8 (0.11)
OCP	*(n = 167)*	23 (0.14)	10 (0.13)	2 (0.11)	11 (0.15)
OHP	*(n = 166)*	22 (0.13)	8 (0.10)	6 (0.33)	8 (0.11)
OFP	*(n = 164)*	21 (0.13)	14 (0.18)	5 (0.28)	2 (0.03)
Any GHA	*(n = 174)*	59 (0.34)	30 (0.36)	7 (0.37)	22 (0.31)
***Verified thrombophilia**, n positive (p)*					
Moderate risk (PC, PS, AT, APS, hmz FVL)		14 (0.08)	6 (0.07)	3 (0.16)	5 (0.07)
Mild risk (htz FVL and/or FII c.20210G>A)		40 (0.23)	18 (0.22)	7 (0.37)	15 (0.21)
Five SNPs (≥ 3 risk alleles)		116 (0.67)	54 (0.65)	15 (0.79)	47 (0.65)
***Biochemical markers**, n positive (n tested)*					
Lupus anticoagulant		7 (120)	3 (66)	2 (14)	2 (33)
Protein C deficiency		1 (167)	0 (79)	0 (17)	1 (71)
Protein S deficiency		1 (143)	0 (79)	0 (17)	1 (47)
Antithrombin deficiency		1 (138)	1 (67)	0 (13)	0 (58)
***Genetic analyses**, EAF*					
Factor V Leiden		0.12	0.11	0.21	0.11
FII c.20210G>A		0.04	0.06	0.03	0.01
Factor XI		0.59	0.60	0.55	0.58
Fibrinogen γ		0.29	0.28	0.32	0.28
ABO non-O		0.49	0.45	0.53	0.51
***Coagulation lab**, median (n) (IQR)*					
PT (INR)		0.9 *(73) 1.0 - 1.0*	1.0 *(41) 0.9 - 1.0*	1.0 *(11) 1.0 - 1.0*	0.9 *(24) 1.0 - 1.0*
Fibrinogen		2.5 *(32) 3.2 - 4.2*	3.3 *(16) 2.5 - 3.3*	4.3 *(3)* - 3.1 *(13) 2.6 - 4.1*	

Table 7. *Thrombophilia investigations. False positive results due to preanalytical factors were excluded. Biochemical results only classified as positive in verified positives (methods)*

AT: Antithrombin, EAF: effect allele frequency, FH: Family History of VTE <50
FVL: Factor V Leiden, LA: Lupus Anticoagulant, n: count, p: proportion, PC: Protein C, PS: Protein S, VTE: Venous thromboembolism

4.2.2 Diagnostic accuracy of a commercial thrombophilia panel

In the evaluation of the commercial clinical/genetic risk algorithm (CGRA) TiC, there were 147 cases (48% female) and 143 controls (73% female), since in the diagnostic accuracy evaluation, the patients with no personal history of VTE (n = 43) were counted as controls together with healthy controls from the blood bank (n = 100).

Variant	VTE n = 147	No VTE n = 143	p value
F5 Leiden	35 (23.81)	22 (15.38)	0.0714
FII c.20210G>A	11 (7.48)	3 (2.10)	**0.0329**
F11	120 (81.63)	120 (83.91)	0.6080
FGG	83 (56.46)	64 (44.75)	**0.0465**
F5 Hong Kong	0	0	
F5 Cambridge	0	0	
F12	68 (46.26)	71 (49.65)	0.5641
F13	58 (39.45)	75 (52.45)	**0.0266**
AT *(II Cambridge)*	4 (2.72)	0	**0.0474**
ZPI *(p.R67X)*	0	0	
ABO A1 haplotype	84 (57.14)	56 (39.16)	**0.0022**

Table 8. *Presence of effect allele variants (≥1 alleles) in individuals with and without previous VTE, N (%). A1 haplotype of ABO blood group; if ≥1 predisposing allele in 3 SNPs.*

Table 8 shows the frequency of risk polymorphisms. The ABO A1 haplotype alleles were significantly more frequent in patients with VTE than without VTE. The presence of at least one risk allele in the genes for prothrombin, antithrombin and the fibrinogen γ chain were also significantly more frequent in patients with previous VTE. The protective allele in the gene for FXIII was significantly less common in patients with VTE. There were no significant differences in the frequency of FVL or the risk alleles in the genes for FXI, FXII or antithrombin. There were no instances of F5 Hong Kong, F5 Cambridge or the nonsense variant in ZPI.

The prognostic characteristics of all genetic risk scores (GRS) and CGRA are shown in Table 9. TiC ® had significantly better discriminative capacity (AUC) than **FVL+F2** (0.71 vs 0.57, $p < 0.0001$), but non-significance in the comparison of only the genes in **TiC** and **FVL+F2** (0.61 vs 0.57, $p = 0.290$). The AUC of **TiC** was significantly higher than **FVL+F2+clinical factors** (0.71 vs 0.65, $p = 0.012$).

The clinical variables alone had a significantly higher AUC than **FVL+F2** (0.660 vs 0.566, p=0.0026). The addition of F11 and FGG to did not significantly improve the AUC of the **TiC GRS** (0.63 vs 0.61, $p = 0.195$) or the **TiC CGRA** (0.65 vs. 0.71, p=0.054).

	FVL+F2	TiC genes	TiC genes +F11+FGG	Clinical	FVL+F2 + clinical	TiC genes + clinical	F11 + FGG + TiC genes + clinical
AUC	0.566 (0.507-0.624)	0.612 (0.553-0.668)	0.628 (0.569-0.684)	0.660 (0.602-0.671)	0.652 (0.594-0.707)	0.706 (0.650-0.758)	0.649 (0.591-0.703)
p value	0.0076	0.0004	0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Sensitivity	30.61 (23.3-38.7)	73.47 (65.6-80.4)	64.63 (56.3-72.3)	74.15 (66.3-81.0)	61.90 (53.5-69.8)	70.75 (62.7-78.0)	68.71 (60.5-76.1)
Specificity	82.52 (75.3-88.4)	45.45 (37.1-54.0)	58.04 (49.5-66.2)	53.85 (45.3-62.2)	65.03 (56.6-72.8)	62.94 (54.5-70.9)	56.64 (48.1-64.9)
LR+	1.75	1.35	1.54	1.61	1.77	1.91	1.58
LR-	0.84	0.58	0.61	0.48	0.59	0,46	0.55
OR	2.08 (1.2-3.6)	2.30 (1.4-3.8)	3.24 (1.4-3.6)	3.34 (2.0-5.5)	4.53 (1.9-4.9)	4.02 (2.5-6.6)	3.82 (2.3-6.3)
OR p-value	0.0097	0.0009	0.0012	<0.0001	<0.0001	<0.0001	<0.0001

Table 9. Prognostic characteristics of the evaluated GRS (left) and GRA (right). AUC: area under the Receiver Operating Characteristic curve (ROC curve), LR+: Positive Likelihood Ratio, LR-: Negative Likelihood Ratio, OR: Odds ratio.

4.2.3 Characterization of a novel prothrombotic variant

The characterization of FII c.1824C>T included 489 cases (370 VTE, 119 cerebrovascular insult (CVI)) of which 190 had family history of thrombosis. The frequency of FII c.1824C>T was increased in patients with VTE and CVI compared to controls and the variant increased the OR for thrombotic events significantly, Table 10. EAF of FV Leiden and FII c.20210G>A were consistent with previous studies in Serbian population [168].

The predictive modelling indicated a change in the mRNA secondary structure; a bulge in the wild-type secondary structure disappeared when a bulge, caused by an unpaired adenosine, disappeared after substitution to uracil (C>T in DNA). The levels of mRNA expression (FII-c.1824T and FII-c.20210A) in transfected cell culture were determined relative to mRNA expression in transfected cells expressing wild-type prothrombin (FII-wt), Figure 18A. Relative levels of FII mRNA expression were significantly increased ($p<0.05$); 1.65 for FII-c.20210A transfectants and 1.64 for FII-c.1824T.

The prothrombin activity measured by clot time was unaffected by FII c.1824C>T, and FII concentration measured by ELISA showed only a non-significant tendency towards increased FII levels. In the sensitive western blot assay, prothrombin levels (detected at 70 kDa) were significantly increased compared to controls ($p<0.05$), Figure 18B. Normalized to standard normal plasma, FII levels were 1.44 ± 0.25 in FII c.1824C>T and 1.63 ± 0.22 in FII c.20210G>A .

Variants	**Controls** *(n = 432)*	**VTE** *(n = 370)*	**CVI** *(n = 72)*
FII c.1824 C>**T**			
Variant carriers, n (EAF)	4 (0.01)	11 (0.03)	5 (0.04)
p-value (vs. controls)		0.038*	0.026*
OR (95% CI)		3.28 (1.04 - 10.39)	4.69 (1.24 - 17.76)
FII c.20210 G>**A**			
Variant carriers, n (EAF)	28 (0.07)	39 (0.11)	3 (0.03)
p-value (vs. controls)		0.040*	0.110
OR (95% CI)		1.70 (1.02 - 2.82)	0.37 (0.11 - 1.25)
FV Leiden G>**A**			
Variant carriers, n (EAF)	16 (0.04)	81 (0.22)	12 (0.10)
p-value (vs. controls)		< 0.0001*	0.007*
OR (95% CI)		7.3 (4.2 - 12.7)	2.9 (1.3 - 6.3)

Table 10. *Variant frequencies and odds ratios in control patients and patient subgroups VTE and CVI. Risk alleles in bold letters.*
**: significance EAF: Effect allele frequency (%), CI: Confidence interval, n: count, p-value: probability value, VTE: venous thromboembolism, CVI: cerebrovascular insult*

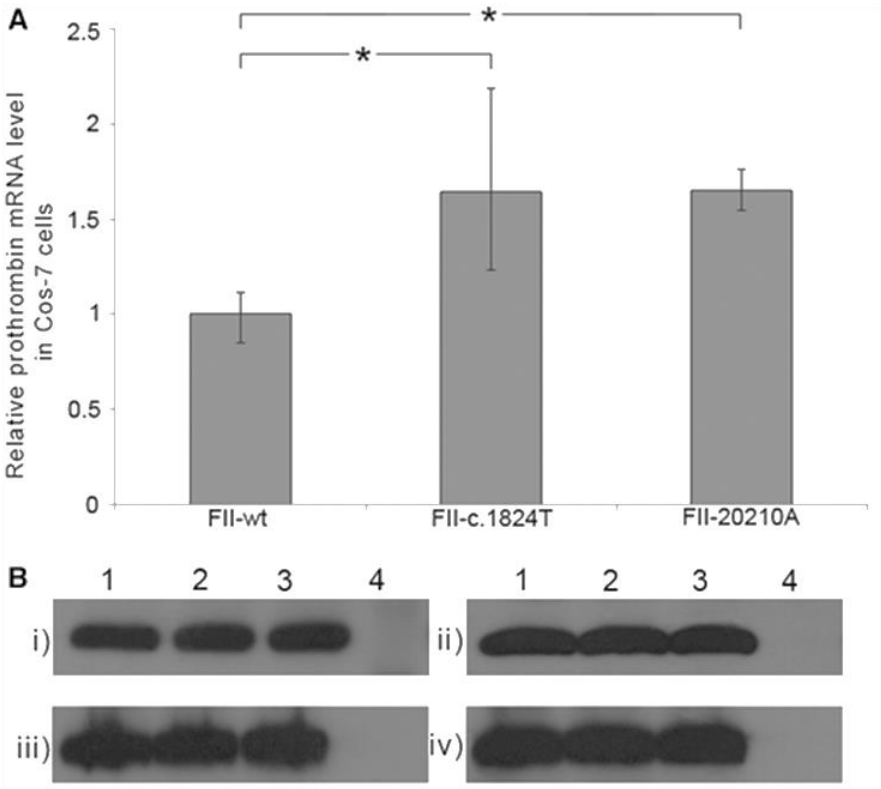
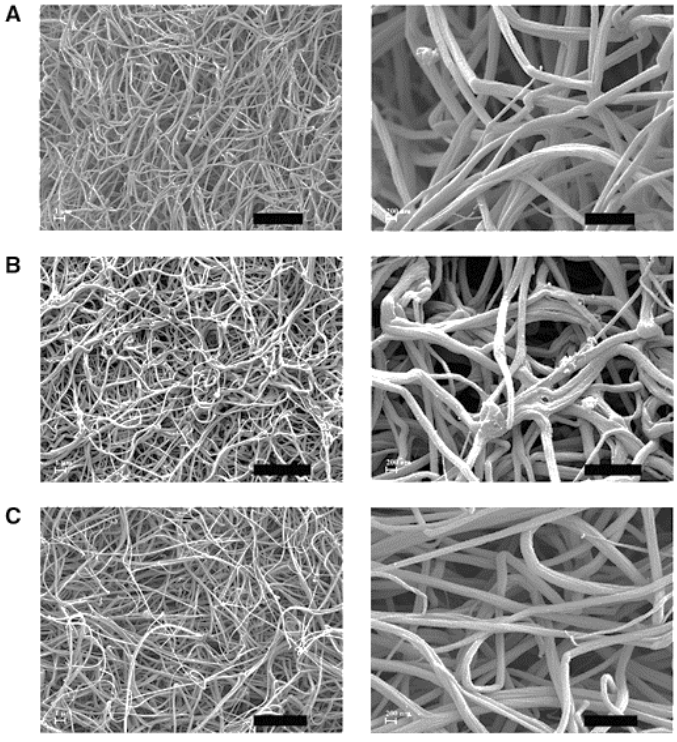


Figure 18. *(A) mRNA expression in FII wild-type vector, and cells transfected with the FII-c.1824T and FII-c.20210A vectors.* $p < 0.05$.(B) Western blot of FII levels in plasma (lanes 1-3: triplicate sample, lane 4: negative control. (i) standard human plasma; (ii) healthy noncarriers; (iii) FII c.20210G>A carriers, and (iv) FII c.1824C>T carriers.*



Scanning electron microscopy of fibrin clots from c.1824C>T carriers revealed denser clots with thinner fibers (median 129 nm, IQR 80 nm) compared to clots formed in plasma of FII c.20210G>A carriers (median 152 nm, IQR 59 nm) and healthy noncarriers (median 159 nm, IQR 72 nm) ($P<0.05$). There were also an increased numbers of branch points and smaller intrinsic pores than in fibrin clots from healthy noncarriers, see figure 19.

Figure 19. *Representative SEM of fibrin clots.*
Black scale marker; 6 μm in left image, 1.2 μm in right
A: Controls, B: FII c.1824C>T , C: FII c.20210G>A .

The routine coagulation assays were unaffected by the variant, except for a significantly decreased mean aPTT, within the reference range (Δ 4s, P<0.05). The OFP was decreased in FII c.1824C>T and clot lysis time was increased, Table 11. In FII c.20210G>A , it was instead the ETP AUC and C_{max} that were significantly increased.

	Healthy controls n=11	**FII c.1824C>T carriers n=6**	**FII c.20210G>A carriers n=6**	**Controls vs. FII c.1824C>T carriers**	**Controls vs. FII c.20210G>A carriers**
Global hemostatic assays, Mean (SD)				***P***	***P***
ETP AUC	87.82 (10.65)	93.67 (13.85)	108.67 (6.50)	0.345	0.001 *
ETP C_{max}	88.91 (6.82)	91.50 (10.39)	114.17 (8.33)	0.542	<0.001 *
OCP	18.98 (5.22)	23.44 (5.68)	21.07 (5.74)	0.123	0.459
OHP	11.01 (4.33)	15.51 (4.67)	13.05 (5.24)	0.065	0.401
OFP	43.40 (8.64)	34.53 (6.66)	39.49 (10.18)	0.046 *	0.414
Coagulation (turbidity), Median (IQR)				***P***	***P***
Lag time (min)	3.60 (2.00)	4.10 (1.80)	3.60 (1.60)	0.960	0.573
Max Abs	0.90 (0.36)	1.14 (0.45)	1.00 (0.44)	0.132	1.000
Clotting rate (slope)	0.08 (0.02)	0.07 (0.02)	0.10 (0.07)	0.615	0.615
Max Abs time (min)	6.40 (3.00)	7.00 (3.25)	5.80 (2.40)	0.338	0.546
Slope time (min)	2.20 (1.20)	2.90 (1.35)	2.10 (1.55)	0.069	0.541
Fibrinolysis (turbidity), Median (IQR)				***P***	***P***
Lag time (min)	4.40 (1.40)	4.40 (1.30)	3.80 (1.35)	0.723	0.312
Max Abs	0.92 (0.41)	1.13 (0.45)	1.03 (0.41)	0.512	1.000
Clotting rate (slope)	0.08 (0.02)	0.08 (0.03)	0.10 (0.06)	0.688	0.421
Max Abs time (min)	7.20 (2.40)	7.10 (2.95)	6.10 (2.25)	0.512	0.246
Slope time (min)	2.60 (0.80)	2.70 (1.65)	2.10 (1.50)	0.358	0.266
Lysis time (min)	23.13 (7.80)	27.40 (7.20)	23.00 (11.85)	s0.044 *	0.687

Table 11. *Parameters of thrombin generation, fibrin aggregation, coagulation times and fibrinolysis times in controls and carriers of FII c.20210G>A and FII c.1824C>T .*

DISCUSSION

4.3 ACUTE VENOUS THROMBOEMBOLISM

Our prospective single-center study supported the use of age-adjusted cutoffs for D-dimer in patients with suspected acute DVT and PE. Age-adjusted cutoff resulted in improved specificity with maintained sensitivity for all evaluated D-dimer assays. We demonstrated that this personalized approach to D-dimer cutoffs lead to a significant decrease in the proportion of patients with false positive results, especially in the older population.

Our objective in the cross-sectional study II was to compare the diagnostic value of D-Dimer to thrombin generation and fibrin aggregation in plasma and FM acute VTE. We could confirm previous findings that ETP AUC and ETP $_{Tlag}$ were significantly increased in acute VTE. However, the discriminatory ability of D-dimer was superior to both FM and the global hemostatic assays. In essence, the global hemostatic assays demonstrated differences between patients with and without acute VTE that were insufficient for effective discrimination and no useful cutoff could be identified for any of the parameters. Moreover, we demonstrated a poor tradeoff between sensitivity and specificity for FM.

Our results further indicated that a large portion of the emergency patients were in a hypercoagulable state, possibly caused by an acute phase reaction, fibrinogen levels were significantly increased regardless of final VTE status. We also demonstrated generally increased coagulation potential (OCP) and decreased fibrinolytic potential (OFP), suggesting that the OHP assay may be unsuitable for exclusion of acute VTE in the emergency department. The use of thrombin generation assays in acute settings may also be prone to acute phase effects which have not been extensively evaluated yet, such as high levels of FVIII in patients with acute phase reactions, which is associated with a true increase in hypercoagulability, but might obscure findings related to acute thrombotic events. Another such issue that affects the accuracy of the thrombin generation assays is the existence of α-2-macroglobulin-thrombin complexes in plasma. It is possible that several of the patients in this study had abnormal levels of α2M, related to other pathology, which may have influenced the results of the ETP assay.

4.3.1 Methodological considerations in study I

The main limitation of the study was the lack of data on clinical pre-test probabilities of VTE in the patients. The study plan dictated that these would be retrospectively collected from medical records in the form of Wells scores. However, in data collection it became

apparent that Wells Scores were documented in a negligible share of the cases and that clinical data for a possible retrospective calculation of Wells scores was not widely available either. The problem of low comprehension of the study among the emergency department personnel including patients for the study could partly be explained by the rapid staff turnover at the emergency department. Since clinical pre-test probabilities could not be defined, the NPVs/PPVs and efficiencies calculated in this study cannot be individually applied as part of a risk algorithm to patients of high or low clinical suspicion This means that results have to be interpreted only on a general level. It might be argued that possibly the lack of documented clinical stratification could mean that assessments of VTE probability were to some extent performed by only clinical *gestalt in the emergency department.* In this case, general accuracy measurements could be of more direct relevance. It has also been suggested that D-dimer (<0.75 mg/L FEU) could be used without previous assessment of clinical pre-test possibility to exclude PE [33], however this has been evaluated in a meta-analysis where it could not be verified [34].

Even though sensitivity was investigated in a group including patients with high clinical probability, sensitivities were high. Furthermore, the false negative results were minor thromboses for all events bar one – i.e. subsegmental PEs and distal DVTs. These minor VTEs are classified as VTE negative in many studies, which increases sensitivity outcomes.

Another key limitation was the fact that only 55% of the patients were investigated by the radiological reference methods. Total coverage would not have been feasible in this study and we decided to instead actively follow all patients in medical records and instruct the patients to return to the ED if symptoms remained. This research practice is not uncommon in evaluations of D-dimer but introduces the possibility of missing false negative cases. To ensure that no missed VTEs were neglected in the study, due to patients deceasing outside of hospital or contacting medical resources not connected to electronic medical records system (TakeCare). We extensively discussed and investigated the retrieval of morbidity and mortality data from national registries, to verify our excluded VTEs. Ultimately, the price and time of such an endeavor were deemed to be too costly.

The limitation of study size was most pronounced for Stagos STA®-Liatest® D-Dimer, resulting in wider confidence intervals for this assay. We chose to include some results from Stago in spite of few samples, since it was possible to discern that the assay seemed comparable to the other three assays.

4.3.2 Methodological considerations of study II

A strength of the study was that samples were handled exactly as real-life samples. We were able to include a large number of patients into the VTE study in just one year, because we could arrange for a study-specific analysis that could be electronically ordered from the medical information system (TakeCare). However, we still noticed a drastic decrease of inclusions to the study from the emergency department when the study had been active for one year, possibly caused by inclusion fatigue or staff turnover. A related problem was that since so many in the staff at the routine coagulation lab were involved in the preanalytical handling, it was very common to forget to aliquot and store samples. Hence, only a limited subset of samples could be analyzed with OHP and ETP in study II (Figure 10). We attempted to decrease the risk of clinical confounders by a rough matching for age before choosing controls at random from the stratified group in order to decrease the risk of selection bias by.

Given our study size it was not possible to stratify patients with PE and proximal or distal DVT. Distal DVT was the main pathology in 32% of the patients with VTE, i.e. cases where surveillance is often recommended over anticoagulant treatment, though the vast majority are treated with anticoagulants [169]. However, our results did not change when we excluded patients with distal DVT from the analysis.

4.4 THROMBOPHILIA INVESTIGATION

In study III, we aimed to explore the hypercoagulable profiles in clinical thrombophilia investigations. Despite frequent anticoagulant treatment, the patients investigated for thrombophilia had increased thrombin generation, fibrin aggregation or decreased fibrinolysis in 34% of cases. This was a discrete increase in hypercoagulable profiles from the conventional thrombophilia panel where 31% of patients had at least one pathological value, but where the majority of positive results were represented by heterozygous FVL or FII c.20210G>A .

Thrombin generation assays and fibrin aggregation measured by turbidimetric assays have been proposed as screening assays for thrombophilia. The secondary aim of study III was to assess whether ETP and OHP could precede the conventional panel in thrombophilia investigations. Notably, the proportion of hypercoagulable ETP and OHP results were low in patients with verifiable thrombophilia and not correlated to the risk level of the thrombophilia. However, inherently hypercoagulable phenotypes were likely obscured by the high prevalence of anticoagulants.

In study V, the recognized problem of thrombotic risk stratification was reflected in the poor predictive abilities of the clinical factors and the evaluated GRS and CGRA. In accordance with previous studies, the ROC AUC of the *clinical factors* was superior to *FVL+F2*, the *TiC genes* and to *TiC+F11+FGG*. The complete *TiC CGRA* had significantly better discriminative capacity than *FVL+F2*, but non-significance in the comparison of the *genes in TiC* vs. *FVL+F2*, which implied that the difference may be attributed to the clinical factors included in the *TiC CGRA*. In spite of geographic differences in EAF, the discriminative ability in our confirmation was similar to that of Soria et al [104], implying that TiC CGRA could be transferable to the Swedish population. As the notion of individual risk stratification without accounting for clinical factors seems clinically inapplicable, the more interesting comparison is that of the CGRA TiC to FVL+F2+ clinical factors. This comparison revealed a discrete but statistically significant increase of AUC in favor of TiC.

In study IV, we could demonstrate a potentially prothrombotic phenotype of the novel sSNP FII c.1824C>T . Our *in-silico* prediction of the RNA secondary structure showed that the C>T change at position c.1824 can be expected to lead to a slight local re-organization of RNA secondary structure. The change of local structure would lead to an increased likelihood of complementary base pairing in the region. Current understanding is that increased complementary base pairing can physiologically prolong the half-life and amount of persisting mRNA. Although prediction of secondary structure is not necessarily a true delineation of intracellular secondary structures, we could demonstrate increased expression of FII mRNA in transfected cells as well as slightly increased FII protein levels in carriers of FII c.1824C>T. Increased FII levels are an established risk factor for VTE, through several mechanisms. Protein expression and concentrations were comparable to the levels seen in FII c.20210G>A . But the increased FII levels seem to exert the prothrombotic effect on the variants by two distinct mechanisms. Increased FII was associated with accelerated and increased thrombin generation in FII c.20210G>A , as well as shortened time to peak turbidity and clotting. The hypercoagulable profile was indicative of a mechanism where the elevated FII levels were associated with faster clotting and increased overall thrombin generation and peak thrombin concentration. In contrast, in FII c.1824C>T carriers, the increased FII levels were accompanied by an increased fibrin generation (OCP) and densely packed fibrin clots, as indicated by increased maximum turbidity [141] and visualized by SEM. We could also demonstrate hypofibrinolysis by decreased OFP and prolonged CLT in carriers of FII c.1824C>T. As described in the background, increased FII levels have been robustly associated with the formation of denser

fibrin clots with thin and highly branched fibers [170], which are in turn associated with resistance to fibrinolysis. The phenotypes have been associated with an increased VTE risk and identified in patients with acute ischemic and hemorrhagic stroke. In light of this, it is very interesting that in our investigation of effect allele frequency, the frequency of FII c.1824C>T was significantly increased in patients with both venous thromboembolism and cerebrovascular insult. Meanwhile, FVL and FII c.20210G>A frequencies were only increased in VTE, consistent with the fact that they have only been robustly associated with venous thrombotic risk. Our findings suggest the possibility that FII c.1824C>T could be a risk factor for CVI and VTE through similar prothrombotic mechanism of action in both conditions.

4.4.1 Methodological considerations of study III

The key limitation of study III was the surprising extent of clinical samples that were collected in suboptimal preanalytical conditions. We had not anticipated that 49% of plasma samples would have been collected while patients were still receiving anticoagulant medication (>80% of patients with history of VTE). Nor did we expect that 50% of patients with history of VTE would be sampled within three weeks of the thrombotic event or that 80% of patients in the FH group would be pregnant at sampling. The groups with different clinical indication for thrombophilia testing varied greatly regarding frequency of current anticoagulant medication [171-173], pregnancy [174], recent VTE [128] and general acute pathology, all transient factors that can influence OHP and ETP results. In short, the FH group consisted mainly of females with family history of VTE that were not treated with anticoagulant medication while the VTE groups, included a large portion of patients receiving anticoagulant medication for a recent thrombotic event.

It seems probable that the low prevalence of hypercoagulable profiles would have been higher if they had been tested without anticoagulant medication, since it has been demonstrated that both thrombin generation and fibrin aggregation are decreased by anticoagulant treatment [171, 172], fibrinolysis has been demonstrated to be slightly increased in dabigatran treatment (OFP) [172]. Conversely, it can be supposed that the wide range of hyper- and hypocoagulable GHA profiles reflected that the patients sampled for thrombophilia investigation while on anticoagulants were in different stages of treatment. It also seems possible that a second explanation for the heterogenous dispersion of coagulation profiles was the high prevalence of patients that were sampled shortly after suffering an acute VTE.

The overlap between positive results by the GHA and the conventional thrombophilia panel was relatively small, given that studies have verified correlations between these two. It seems probable that the transient interference of anticoagulant medication resulted in decreased GHA profiles in those patients with verifiable thrombophilia. Unfortunately, there were not enough samples without anticoagulants to reliably calculate the sensitivity or specificity of the global hemostatic assays to detect verified conventional thrombophilias.

The converse relation, of transient hypercoagulability caused by pregnancy, is a probable explanation for the relatively frequent hypercoagulable result in the FH group and in patients without verified conventional thrombophilia. It is noteworthy that so many interfering factors were present in this descriptive study that was conducted solely in samples that have been used for clinical decision making.

4.4.2 Methodological considerations in study IV

The major limitation of study IV was the small number of FII c.1824C>T and c.20210G>A carriers recruited for the phenotype characterization. This was due to the low frequency of FII c.1824C>T in thrombotic patients, and the fact that in order to attain interpretable experiment results, we required investigated carriers to be completely without anticoagulant therapy. The small sample size may be the cause of some trends not achieving significant difference. The issue of insufficient power will be mitigated by the impending confirmation study in the KUL thrombophilia cohort. Another limitation which cannot be dismissed, considering that thrombosis is a multifactorial disorder, is the possible effect of unattributed confounding risk factors. Future studies addressing these limitations will contribute to the impact of the findings of this study.

Despite small number of cases, western blot confirmed that FII c.1824C>T leads to a slightly increased concentration of prothrombin in carriers, although we did not detect an increase of FII activity. Poor correlation between prothrombin activity and concentration has also been demonstrated in carriers of FII c.202010G>A [175] and is likely explained by dysfunctional variants of prothrombin and *in-vivo* modulators of prothrombin activation and thrombin function. Coagulation times were not decreased in either of the FII variants, which is expected, since the maximal initial thrombin generation rate is attained in normal plasma concentrations of prothrombin.

4.4.3 Methodological considerations in study V

The comparison of TiC and *F5+F2+clinical factors* revealed a discrete but statistically significant increase of AUC in favor of TiC. However, notwithstanding the significance of the ROC AUC differences, the clinical significance is uncertain. It seems improbable that a 0.05 increase in AUCs to 0.7 could be translated into clinical value. Perhaps related to this concern, a cost-effectiveness analysis has been published [176]. The analysis was based on two case-control studies, since there are to date no prospective studies evaluating the effect of TiC as an intervention with VTE as the outcome.

We need to acknowledge that the study was not powered to determine effect allele frequencies in the Swedish population, nor were the study participants a representative sample of the Swedish population. The frequencies of risk alleles were not a primary endpoint of this study, and were, as such, only presented for increased comprehensibility of the ROC results.

The use of ROC in a non-prospective study should be commented. It is frequent in genetic diagnostic accuracy studies to calculate discriminatory ability by ROC and sensitivity/ specificity using thrombotic occurrences in patient history as though samples were prospectively collected. In the ROCs of study V, the different cutoffs represent number of variants for the evaluated GRS, with weights as per the TiC risk coefficients (Table 3).

4.5 STUDY MODEL

The approach that we used to collect study samples via the clinical chemistry laboratory is a compelling model to efficiently power a study. Considerable data can be obtained from laboratory information systems and analysis of left-over plasma without additional collection of research samples. However, it is important to be aware of the weaknesses of this method.

The model is very sensitive to confounders, since these cannot be effectively managed in the inclusion process. A possible way to increase the specificity of inclusions is to form close collaborations with clinicians. If inclusions are done prospectively, there is an opportunity to gather prospectively collected data (eg. Wells Score). This necessitates a strict adhesion to GCP with detailed protocols and comprehensive start up meetings.

A juxtaposed risk, which is present if patients are invited by letter is that extensive exclusions based on only information in the consent form could lead to a risk of small study samples. A strategy to prevent this could be to store samples from all consenting patients

and save exclusions to a later time-point. Continuous invitations, inclusion assessments and bio-banking are also laborious to perform in parallel with routine operations. Finally, if left-over plasma samples are used, there is an appreciable risk of low sample volumes which the necessitates strict prioritizing of investigations in the samples.

5 CLINICAL IMPLICATIONS & FUTURE PERSPECTIVES

5.1.1 Optimizing the use of D-dimer

D-dimer is the only biomarker in routine clinical use for VTE, and the low specificity represents a significant problem. Increasing the effectiveness of the diagnostic process would be a clinical and economic breakthrough, especially considering the global disease burden of patients suffering from VTE. Study I added to the significant efforts of recent years that support age-adjusted cutoffs for D-dimer in DVT and PE. Our evaluation demonstrated that an adjusted cutoff resulted in a significant reduction in false positive results without decreased sensitivity, which reflects avoidable health risk, prolonged emergency department wait times and cost of imaging techniques without any compromise of clinical safety. We demonstrated that the unwarranted imaging to rule out VTE could be avoided in >5% of all patients with suspected disease, while in elderly patients as many as 20% could avoid unnecessary imaging.

The advantages of age adjusted D-dimer cutoffs are not uncontroversial [177] and the concept has also been challenged in a few recent studies These studies have shown an equal improvement of specificity and proportion of tests with negative results between age adjusted cutoffs vs. general increase of cutoffs in a low risk population with a corresponding low prevalence of VTE at 6.6 % [57] and 7.8 % [56, 58] for the studies respectively. Our investigation of ROC curves demonstrated that the sensitivity of the D-dimer assays is rather resistant to changes of the cutoff if the cutoff is kept below 1 mg/L FEU, displayed in the cumulative data analysis curves (Figure 13). We interpret the findings of the mentioned studies as a symptom of this observation. In a population of low clinical probability, it is not surprising that the cutoff can be increased within this comfort zone without affecting sensitivity if the study population is not large enough. Since all utilization of D-dimer rests on the foundation of a reliably high sensitivity, a decrease of the robustness of the sensitivity could have negative consequences which would not be apparent before such an increase would be tested in a large prospective study.

Instead the opposite course of action could be pertinent, i.e. a further individualization of the D-dimer cut-offs. Using the Tina-quant D-Di we revealed that in patients < 50 years (n=346), sensitivity was 0.91 and specificity 0.82. This is considerably lower than the CLSI requirements for sensitivity of D-dimer and raises the question whether the general cutoff is set too high for patients younger than 50? Regrettably, our study was not powered to determine a suitable cutoff for only patients < 50 years, but the issue was recently

addressed in a study demonstrating that the lowest levels of sensitivity of D-dimer were found in female patients, unprovoked DVT, low thrombotic burden, and distal DVT [178]. A fixed D-dimer cutoff 0.25 mg/L FEU in patients < 60 was suggested. In our cohort, applying this cutoff to the D-Di Tina-quant would result in sensitivity 0.97 and specificity 0.52, which is a comparable to the specificity at 0.50 mg/L FEU in 60–70-year-old patients.

Increasing the effectivity of the VTE diagnostic process has potentially major beneficial clinical and economical effects on the management of VTE. Because of the close association between the research group and the Karolinska clinical chemistry laboratory, clinical implementation of our findings could be more easily accomplished. Age-adjusted D-dimer cutoffs were clinically applied in Stockholm in late 2017 and have since been introduced in many parts of Sweden. The aim was that the fewer false positive results would enable diagnosis to become faster, cheaper and possibly result in decreased health risks from intravenous contrast, radiation and unnecessary hospital admissions and short-term anticoagulation. A descriptive follow-up study of the clinical outcome of the implementation is underway, in collaboration between the PhD student and the Department of Medicine at Karolinska Institutet. Preliminary data from a small-scale evaluation of the implementation in Region Västmanland, was presented at the annual meeting of clinical chemistry in 2019; suggesting safe implementation and decreased false positive results.

5.1.2 Global hemostatic assays in the diagnosis of venous thromboembolism

As previously stated, there is significant room for improvement of the biochemical diagnosis of venous thromboembolism. However, identifying an assay superior to D-dimer has proven to be a challenge. Although some data point to the potential usefulness of the global hemostatic assays, a lack of standardization has hampered progress. Moreover, the diagnostic accuracy of ETP and OHP for acute VTE had not been assessed. The results of our study were in line with recent clinical evaluations of other thrombin generation assays for acute VTE. Our results indicated that neither the ETP nor the OHP assay would be clinically useful additions as biomarkers for the diagnosis of acute VTE in the emergency department. The evaluation of Soluble Fibrin for acute VTE, demonstrated that the increased diagnostic specificity of Soluble Fibrin in comparison with D-dimer came at the generally unacceptable cost of a decreased sensitivity. The study further implied that OHP and ETP were sensitive to hypercoagulability possibly caused by acute phase effects and comorbidities that may well be unavoidable in outpatients at the emergency department.

During the course of this thesis, the samples have been used in additional evaluations of feasible biomarkers of VTE which have also struggled with improving the diagnostic trade-off of sensitivity and specificity beyond D-dimer. These, still unpublished findings of other biomarkers of acute VTE seem to implicate that a limited specificity for thrombotic events leads to similar assay difficulties as in D-dimer, ETP and OHP, that is high false positive rates in acute VTE. Since fibrin deposition in tissues in the absence of acute thrombotic events are common, it would perhaps be more fruitful to pursue highly specific biomarkers of VTE in the pathophysiological pathways initiating VTE. It is however operative to keep in mind that there are several triggering mechanisms and a possibility could be a partitioning between thrombosis and thromboembolism of various origin and the use of distinct biomarkers to diagnose acute VTE.

5.1.3 Global hemostatic assays in the risk stratification of thrombophilia

Study III was a cross-sectional descriptive study aimed to be a real-world pilot of the diagnostic landscape associated with expanding the current thrombophilia panel to include global hemostatic assays, which are emerging as general biomarkers of thrombotic risk. Knowledge of the diagnostic landscape can be used to strategically optimize implementation of the assays, not only from a clinical chemistry perspective.

Addition of the ETP and OHP to the conventional thrombophilia panel led to an increased yield of objectively hypercoagulable results but did not evaluate how they should be interpreted in the thrombophilia investigation. The study accentuated that the ETP and OHP will only convey a snapshot of the patients' coagulation profile. This makes proper sampling a very important issue if the GHA were to be clinically implemented for risk prediction of first or recurrent VTE.

It is already known that the global hemostatic assays should be taken with the utmost attention to preanalytical details, but this study adds gravity to that knowledge. The possibility that ETP and OHP can risk false negative results in patients using anticoagulant medication and false positive results in patients suffering a recent VTE, may pose an obstacle to their correct clinical use as risk markers for recurrent VTE. The investigation of risk for first time VTE might be associated with less preanalytical pitfalls.

In light of these examples, it is reasonable to assume that the global hemostatic assays will also risk improper sampling, whether it be due to lack of knowledge or with the expert knowledge to interpret results related to preanalytical sources of error.

The study highlighted difficulties of result interpretation in samples taken in suboptimal preanalytical conditions. Further prospective trials are needed to directly address if, how and when the global hemostatic assays could potentially be used as clinical biomarkers of thrombotic risk. Special attention should be directed to guiding proper clinical utilization of the assays, related to for example influence from anticoagulant medication, recent thrombotic events and acute phase reactions.

A relevant parallel seems to be the correct utilization of the lupus anticoagulant assay, an assay that is commonly subject to pre-pre-analytical errors. For example, more than half of lupus anticoagulant testing in this cohort had been ordered in patients currently using anticoagulant medication. A considerable proportion of thrombophilia investigations was also initiated within a few weeks of diagnosis and treatment for acute VTE. The lacking adherence to pre-pre-analytical recommendations could well indicate an uncertainty as to when and how thrombophilia investigation and lupus anticoagulant are optimally tested. It may however instead reflect an informed practical strategy, aimed at shortening the turnaround times of investigation. Further research and clinical recommendations on the proper utilization of plasma based GHA related to anticoagulant medication and recent VTE would be useful.

If the ETP and OHP were to be useful as part of investigation of the risk of a first or recurrent venous thrombosis, diagnostic sensitivity and clinical cut off values would most likely need to be further examined instead of using the 95th percentile normal ranges. Naturally, the determination of clinical cutoff values would need to be chosen with regard to the clinical intentions – whether a sensitivity could be reached, where the ETP or OHP could be used for initial screening of hypercoagulability and guidance of further thrombophilia investigation.

Since the GHA parameters reflect somewhat different aspects of coagulation, an interesting prospect is that patients with different underlying hypercoagulability could possibly benefit from risk assessment by different GHA parameters. Future prospective studies comparing thrombin generation and fibrin aggregation assays would be needed to determine if one or both of these modalities would give the best clinical value.

The highest proportion of hypercoagulable results in one parameter was seen in patients with a personal history of VTE combined with a family history of VTE, where 28% presented hypofibrinolytic profiles with decreased OFP. OFP may for example be the parameter most suited to detect thrombotic conditions where impaired fibrinolysis is an

important contributor to the hypercoagulable state, such as APS [146, 151, 179] or obstetrical patients [143]. This study included only two APS patients without anticoagulation and hence cannot substantiate such a hypothesis, only hint at tendencies in different subgroups. It is worth noting that decreased fibrinolysis was at least as common as increased thrombin generation or fibrin aggregation in this study, and most common in patients with personal history of VTE in combination with family history of VTE. With this in mind, it would be interesting to further investigate clinical conditions related to decreased fibrinolysis.

Likewise, an investigation of personalizing anticoagulant treatment according to hypercoagulability profiles would be of an interesting prospect and a are being investigated for at our laboratory adapted to the treatment of hemophilia patients . A possible future rationale for analyzing a combination of GHA could perhaps be guidance of individualized anticoagulation medication for patients with combined hypercoagulable risk factors but with predominantly hypofibrinolytic or hypercoagulable tendencies. Current evidence point to DOACs as less suitable for prophylaxis in APS patients [180, 181], in concurrence with the data suggesting that APS is partly related to impaired fibrinolysis [151].

5.1.4 Prothrombotic variants as genetic markers of thrombophilia

Addition of three additional prothrombotic SNPs in (Fibrinogen γ-chain (FGG), Factor XI (FXI) and the ABO blood group gene (ABO)), has been shown to improve the risk prediction for first time VTE and for recurrent VTE, with the risk increasing in proportion to the number of risk alleles.

The clinical evaluation of additional prothrombotic polymorphisms in suspect thrombophilia, will be implemented into the clinical thrombophilia investigation panel within six months if the polymorphisms prove useful.

Clinical genetic risk algorithms for VTE have the potential to improve the predictive ability of the classical genetic thrombophilia markers, but we could only demonstrate a slight improvement of ROC which is of uncertain clinical value. However, it should be acknowledged that a prospective advantage of TiC is that a CGRA is potentially easier to implement correctly, than an individual medical expert assessment, taking into account the relative importance of different clinical and genetic risk factors and possible interplays. In study III, some worrying indications of pre-pre-analytical difficulties were suggested, it might therefore be hypothesized that TiC could present a higher clinical value for the unaccustomed thrombophilia investigator.

5.1.5 Silent gene variants as attributors of thrombophilia heritability

We explored a novel prothrombotic sSNP in the gene for prothrombin and found that it leads to increased expression and plasma levels of prothrombin, decreased fibrinolysis potential, prolonged clot lysis time and the formation of highly stabilized fibrin clots. This novel risk factor for thrombosis points to sSNPs as an uncharted component of unexplained thrombophilia heredity. We will continue to explore FII c.1824C>T, by further phenotype characterization and investigation of variant frequency in the KUL thrombophilia cohort.

The confidence in FII c.1824C>T as a prothrombotic SNP would be strengthened if it would be identified in GWAS. A recent meta-analysis did not identify FII c.1824C>T as a potential risk factor of VTE [182]. It has since been elucidated that risk-stratification for venous thromboembolism should be treated as two distinct risks; that of first VTE and that of second VTE. FII c.1824C>T is only suggested as a risk factor of first VTE.
An established weakness of GWAS is their low ability to identify relevant variants with an EAF <0.01 as well as common variants associated with mild effects. In addition, synonymous variants are disregarded in VTE GWAS. Given the evidence that corroborate the role of sSNPs in VTE pathophysiology, whole exome sequencing with acknowledgement of potential sSNPs would decidedly be preferable in future genetic explorations of thrombotic risk.

5.1.6 Thrombophilia testing

As discussed earlier, the future of thrombophilia testing is uncertain. Conventional thrombophilia investigation has not been shown to improve outcomes in tested patients. Indeed, it may be unsurprising that the analysis of two mild genetic risk factors together with a handful of very uncommon anticoagulant deficiencies does not give population-scale impact on outcomes and that isolated testing of APS is the main thrombophilias testing currently recommended by several experts. However, it seems backward to not further investigate the potentials of personalized health care for VTE, which is an important global cause of morbidity and mortality, just as we are emerging into the digitalized era. It seems more probable that we will be able to elucidate complex diseases such as VTE than ever before, as machine learning solutions may be required to interpret data from whole exome explorations, miRNA, SNP-SNP-interactions and other mechanisms still unknown to us.

6 CONCLUSIONS

6.1 STUDY I

A prospective single-center study indicating that age-adjusted D-dimer cutoffs can eliminate false positive results in more than 5% of all patients with suspected VTE. In elderly patients with suspected VTE the decrease in false positive results could even affect as many as 20% of patients. Results were valid for four different reagents.

6.2 STUDY II

A cross-sectional diagnostic study where the OHP and Innovance® ETP assays did not improve the diagnosis of acute VTE in emergency department outpatient.

The study suggested that many patients are in a hypercoagulable state when contacting the emergency department for suspected VTE, due in part to an increased fibrinogen levels.

6.3 STUDY III

A cross-sectional descriptive study indicating that at least a slightly increased proportion of patients could be given graduated risk assessments based on the analysis of thrombin generation and fibrin aggregation.

The study highlighted some difficulties of result interpretation in samples taken in suboptimal preanalytical conditions, present in the samples that had been clinically used for laboratory investigation of each patient.

6.4 STUDY IV

A translational study, elucidating the functional effects of a newly identified synonymous polymorphism. The study could corroborate the FII c.1824C>T variant as a feasible contributor to thrombotic risk by functional explorations of coding DNA, via mRNA and proteins to a comprehensive overview of the hemostatic profiles in carriers of the sSNP.

6.5 STUDY V

A cross-sectional diagnostic study indicating that a clinical-genetic algorithm developed in Spain had similar discriminative ability in a nonpregnant Swedish population sample, despite significant differences in the allelic frequencies of included genetic variants.

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