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Thrombin generation in different cohorts

Evaluation of the haemostatic potential

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Cover: Computer model of a molecule of the protein thrombin.
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For my parents and Christina;
thank you for everything

Για τους γονείς μου και τη Χριστίνα:
ευχαριστώ για όλα

I must create a system, or be enslaved by another man's.

– *William Blake*

Keep your beliefs balanced and go on.

– *Giannis Chairetis*

Abstract

The aim of this thesis is to evaluate thrombin generation in patients with thrombophilia (Paper I), in patients with venous thromboembolism (Paper II), in healthy women during the menstrual cycle (Paper III), in patients with liver disease (Paper IV) and in patients with mild deficiency of factor VII (Paper V).

For this purpose, thrombin generation was measured in platelet poor plasma by the calibrated automated thrombogram (CAT®) assay. Thrombin generation expresses the overall haemostatic potential, in contrast to the more traditional coagulation tests, which concentrate on individual factors or coagulation pathways. The thrombin generation markers that were measured and studied were: lagtime (clotting time), endogenous thrombin potential (ETP, total thrombin concentration), peak (maximum thrombin concentration) and time to peak (ttpeak).

The cohorts for Papers I and II are part of a larger cohort (The LInköping Study on Thrombosis, LIST), which included 516 consecutive patients who presented at the Emergency Department of Linköping University Hospital, Sweden with the clinical suspicion of venous thrombosis. In Paper I thrombin generation was measured in the absence of thrombomodulin in patients with thrombophilia (factor V Leiden, n=98 and prothrombin G20210A mutation, n=15) and in an equal number of age- and gender-matched controls. The results were associated with the presence of thrombosis, as well as gender and age. It was shown that thrombin generation did not differ significantly among patients and controls. Patients with and patients without thrombophilia who had suffered a thrombosis upon inclusion had longer lagtime compared with their counterparts without thrombosis. Neither age nor gender had any effect on the results.

In Paper II, thrombin generation at the time of an acute thromboembolic episode was studied as a potential early marker for recurrence during a 7-year follow-up in 115 patients with venous thrombosis upon inclusion. It was shown that patients with recurrences during follow-up had longer lagtime and ttpeak at the time of the acute thrombosis, whereas those without recurrences had higher ETP and peak. Those results were particularly evident in the group of patients with an unprovoked thrombosis upon inclusion.

In Paper III, thrombin generation was measured in the follicular and luteal phase of a normal menstrual cycle in 102 healthy women not taking oral contraceptives. The results were associated with haemostatic parameters (fibrinogen, antithrombin, D-dimer, plasminogen activator inhibitor-1, factors VII, VIII, X and von Willebrand) as well as the physiological concentrations of oestradiol, progesterone, antimüllerian hormone and sex hormone-binding globulin and the number of pregnancies and deliveries for these women. ETP

was significantly higher during the luteal phase. However, this could not be explained by the elevation of other procoagulant factors during the same phase. Progesterone was found to exert a more significant effect on haemostasis than oestradiol during both phases (multiple regression analysis).

In Paper IV, thrombin generation was measured in the presence and absence of thrombomodulin in 47 patients with portal vein thrombosis, PVT (11 with cirrhotic PVT and 36 with non-cirrhotic PVT), 15 patients with Budd-Chiari syndrome and 24 patients with cirrhosis, as well as 21 healthy controls. Since 15 patients with PVT (2 with cirrhotic PVT and 13 with non-cirrhotic PVT) and 10 patients with Budd-Chiari syndrome were treated with warfarin at the time of the blood sampling, an equal number of patients matched for age, gender and prothrombin time-international normalized ratio with atrial fibrillation and no hepatic diseases were used as controls. It was shown that hypercoagulability, expressed as total and maximum concentration of generated thrombin as well as thrombomodulin resistance [thrombin generation markers measured in the presence]/[thrombin generation markers measured in the absence of thrombomodulin] was pronounced in the groups of patients with cirrhosis, regardless of the presence of splanchnic thrombosis.

In Paper V, thrombin generation in the presence of human and different concentrations of rabbit thromboplastin was measured in 10 patients with mild deficiency of factor VII and in 12 controls. In these patients, the levels of factor VII varied slightly depending on the origin of the thromboplastin used in the reagent. Nine out of 10 patients had a mutation in common (Arg353Gln), which was, however, not associated with the diversity in the factor VII measurements due to the origin of thromboplastin. ETP in patients with mild factor VII deficiency was about 86% of the ETP in the control group. The expected thrombin generation patterns with increasing concentrations of thromboplastin did not differ depending on the origin of thromboplastin in the patient group.

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Papers

Abbreviations

α2M	alpha 2 macroglobulin
AMH	antimüllerian hormone
APC	activated protein C
aPTT	activated partial thromboplastin time
AUC	area under the curve
BCS	Budd-Chiari syndrome
BMI	body mass index
C-PVT	cirrhotic-PVT
CAT®	Calibrated Automated Thrombogram
cd	cycle day
CLD	chronic liver disease
CP	Child Pugh
CTI	corn trypsin inhibitor
DVT	deep vein thrombosis
ETP	endogenous thrombin potential
F1+2	prothrombin fragment 1+2
FII	factor II
FIX	factor IX
FIXa	activated factor IX
FV	factor V
FVa	activated factor V
FVII	factor VII
FVIIa	activated factor VII
FVIII	factor VIII
FVIIIa	activated factor VIII
FX	factor X
FXa	activated factor X
FXI	factor XI

FXIIa	activated factor XII
FXIII	factor XIII
hs-CRP	high sensitivity C-reactive protein
LH	luteinizing hormone
LMWH	low molecular weight heparin
NC-PVT	non-cirrhotic PVT
OC	oral contraceptives
PAI-1	plasminogen activator inhibitor-1
PAR	protease activated receptor
PC	protein C
PE	pulmonary embolism
PPP	platelet poor plasma
PRP	platelet rich plasma
PT	prothrombin time
PT-INR	prothrombin time-international normalized ratio
PTG20210A	prothrombin G20210A
PVT	portal vein thrombosis
SHBG	sex hormone-binding globulin
TAT	thrombin-antithrombin complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
TP	thromboplastin
ttpeak	time to peak
VTE	venous thromboembolism

List of papers

- **Chaireti R**, Jennersjö C and Lindahl TL. Thrombin generation and D-dimer concentrations in a patient cohort investigated for venous thromboembolism. Relations to venous thrombosis, factor V Leiden and prothrombin G20210A. The LIST study. Thromb Res 2009 Jun;124(2):178–84
- **Chaireti R**, Jennersjö C and Lindahl TL. Is thrombin generation at the time of an acute thromboembolic episode a predictor of recurrence? The LInköping Study on Thrombosis (LIST) – a 7-year follow-up. Thromb Res 2013 Feb;131(2):135–9
- **Chaireti R**, Gustafsson KM, Byström B, Bremme K and Lindahl TL. Endogenous thrombin potential is higher during the luteal phase than during the follicular phase of a normal menstrual cycle. Hum Reprod 2013 Jul;28(7):1846–52
- **Chaireti R**, Rajani R, Bergquist A, Melin T, Friis-Liby I-L, Kapraali M, Kechagias S, Lindahl TL and Almer S. Increased thrombin generation in splanchnic vein thrombosis is related to the presence of liver cirrhosis and not to the thrombotic event.

  Submitted to Liver International.
- **Chaireti R**, Arbring K, Olsen OH, Persson E and Lindahl TL. Thrombin generation and levels of factor VII activity measured in the presence of rabbit and human thromboplastins in patients with mild factor VII deficiency – effects of mutations in factor VII.

  In manuscript.

Part I

Thrombin generation: the method

1. Thrombin: structure and functions
2. Thrombin generation
3. Thrombin generation assays

1 Thrombin: structure and functions

1.1 Thrombin structure and interaction with Na^+

In 1872, Alexander Schmidt described thrombin as the enzyme converting fibrinogen into fibrin and named it initially "fibrinferment" [1]. Thrombin is the activated form of prothrombin, or factor II (FII) [2]. The gene for prothrombin (thrombin) is located in the eleventh chromosome (11p11-q12) [3].

Thrombin is a Na^+ activated, allosteric serine protease of the chymotrypsin family. It bears the chymotrypsin-like fold where two 6-stranded β-barrels come together asymmetrically to host at their interface the residues of the catalytic triad H57, D102 and S195 (Figure 1). Thrombin is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) residues that are covalently linked through a disulfide bond between residues C1 and C122. The B chain hosts the entrance to the active site and all known active epitopes of the enzyme [4]. The A chain, which is placed in the back of the molecule, is considered an appendage of the activation process from prothrombin. However, natural variants of the prothrombin molecule including the A chain are associated with severe bleeding [5].

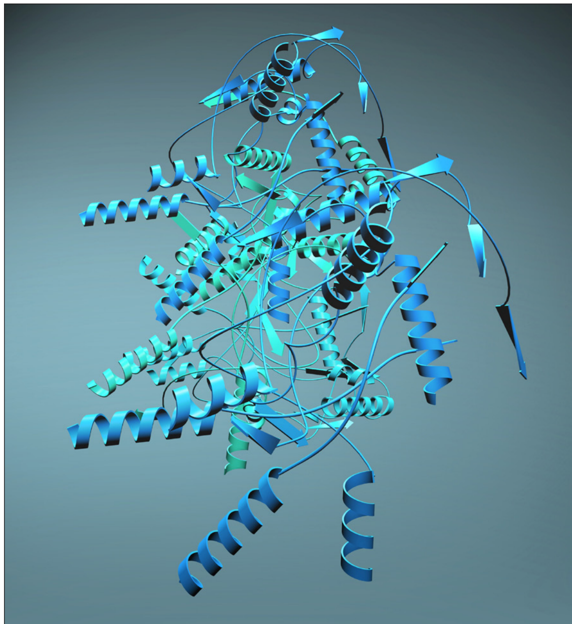

FIGURE 1. *Structure of the thrombin molecule. (Source: http://www.123rf.com. © Elena Pankova.)*

One of the most interesting features of thrombin is its interaction with Na^+. This binding is necessary for the effective cleavage of fibrinogen and the activation of factors V (FV), VIII (FVIII) and XI (FXI), which play a central role in the generation of thrombin in the coagulation cascade. However, Na^+ is not important for the activation of protein C (PC). That shows that Na^+ binding promotes only the procoagulant effect of thrombin [6, 7].

Na^+ binding converts thrombin from the low activity slow Na^+ free thrombin to the high activity fast Na^+ bound form. Interestingly, hypernatremia has been associated with venous thrombosis, especially in patients with diabetes. The association between bleeding and hyponatraemia has not been as accurately documented, because one of the causes of hyponatraemia is the presence of subdural haematoma [7].

Na^+ binding also promotes the prothrombotic and signaling functions of the enzyme by cleaving protease activated receptor (PAR)-1, PAR-3 and PAR-4. PAR-1 and PAR-4 are the receptors that trigger platelet activation and aggregation in humans, thus mediating the procoagulant function of thrombin [8, 9].

1.2 Functions of thrombin

Thrombin has two opposing functions; it acts both as a procoagulant and as an anticoagulant. As a procoagulant, thrombin leads to conversion of fibrinogen to an insoluble fibrin clot, which is essential for the fastening of platelets at the site of the wound (lesion) and the initiation of the healing process. Thrombin exercises its anticoagulant role by binding to thrombomodulin at the intact endothelium and subsequently activating PC. The thrombin-thrombomodulin interaction decreases the ability of thrombin to cleave fibrinogen, but enhances the affinity of the enzyme towards PC. Activated PC (APC) inactivates the activated forms of factors V (FVa) and VIII (FVIIIa), two essential cofactors for the activated factors X (FXa) and IX (FIXa), thereby downregulating thrombin generation [10].

Additionally, thrombin has a central role in inflammatory response, is important for embryonic vascular development, angiogenesis, tissue repair after a trauma (recruitment/activation of inflammatory cells, revasculization, progress of the healing process), neurodegeneration and neuroprotection, and tumor biology and tumor enhancement by stimulating tumor neoangiogenesis and increasing the presence of tumor cells in the circulation [11].

2 Thrombin generation

2.1 Overview of thrombin generation

The haemostatic mechanism leading to thrombin formation involves three procoagulant vitamin K-dependent enzyme complexes [FIXa, FXa, activated factor VII (FVIIa)] and one anticoagulant vitamin K-dependent complex. Each complex is composed of a vitamin K-dependent serine protease and a cofactor protein. The plasma proteins are inactive forms and require activation before they can participate in the biological process. The protease-cofactor complex assembles on a membrane surface provided by activated platelets or damaged cells [12, 13].

Thrombin is generated via a two-phase procedure: initiation and propagation. In the initiation phase, only tiny amounts of thrombin are produced (Figure 2). Those amounts are, however, essential for the next phase (propagation) during which the bulk of thrombin is produced (Figure 3). The event that triggers thrombin generation is the interaction between tissue factor (TF), which is exposed at the site of vascular injury, and FVIIa. FVIIa exists in plasma at a concentration of ≈1–2% of total factor VII (FVII). The FVII zymogen is cleaved at Arg152 by a group of proteases (thrombin, FIXa, FXa, as well as the complex FVIIa-TF), which leads to activation of FVII to FVIIa. FVIIa is resistant to antithrombin, and thus preserved in the plasma, as it can act as a catalyst only when it forms a complex with TF [14]. The FVIIa-TF complex catalyzes the activation of both factor IX (FIX) and factor X (FX), the latter initially being the more efficient substrate [13]. However, FIX is important, as it is not inactivated by the tissue factor pathway inhibitor (TFPI) and can further induce the coagulation process [15]. The initial FXa activates tiny amounts of prothrombin to thrombin; those tiny amounts of thrombin are essential to the acceleration of the process by activating platelets, factor V (FV), and factor VIII (FVIII) [13]. Upon formation of FVIIIa, the FIXa generated by the FVIIa-TF complex combines with FVIIIa on the activated platelet membrane forming the intrinsic factor Xase that is the major activator of FX. The FVIIIa-FIXa complex is 10^5–10^6-fold more active than FIXa alone and ≈50 times more efficient than FVIIa-TF in catalysing the activation of FX [16, 17]. The majority of FXa is produced by the intrinsic factor Xase. FXa combines with FVa on the activated platelet membrane surface forming the prothrombinase complex, which converts prothrombin to thrombin during the propagation phase. Prothrombinase is 300,000-fold more active than FXa in catalysing prothrombin activation. The major bolus (≈96%) of thrombin is produced during the propagation phase of the reaction [16, 17].

The formation of a fibrin clot, which is also the end point in most coagulation assays, occurs when 10 to 30 nmol/L thrombin or ≈3% of the total amount of thrombin is produced during the reaction, which is provided by only ≈7 pM prothrombinase [18].

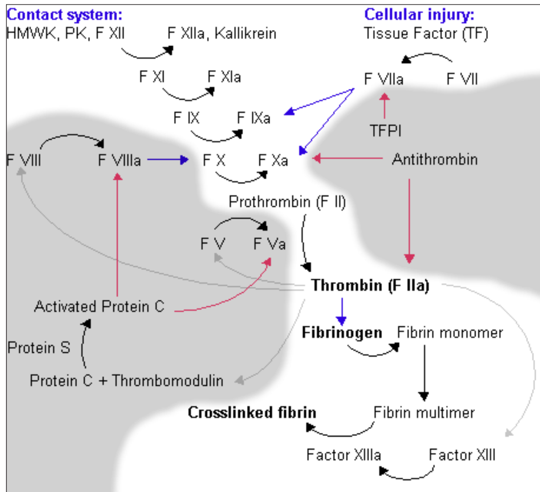

FIGURE 2. *A graphic presentation of the coagulation system illustrating the initiation phase of thrombin generation (in white). (Source: http://en.wikipedia.org/wiki/Image:Coagulation_cascade.png. Image modified by Jonas Walldén.)*

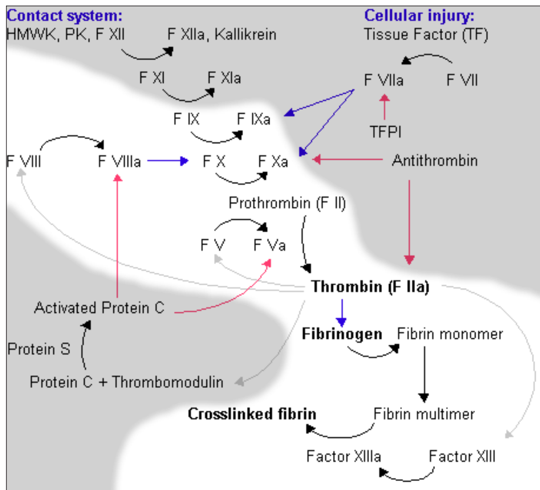

FIGURE 3. *A graphic presentation of the coagulation system illustrating the propagation phase of thrombin generation (in white). (Source: http://en.wikipedia.org/wiki/Image:Coagulation_cascade.png. Image modified by Jonas Walldén.)*

2.2 Inhibition of thrombin generation

Thrombin production is largely regulated by TFPI, antithrombin and PC (Figure 4). TFPI acts by blocking the TF-FVIIa-FXa product complex, which in its turn neutralizes the extrinsic factor Xase.

The amount of antithrombin in plasma is twice or more the amount of the enzymes generated by the TF pathway. Antithrombin is a highly effective neutralizer of the mature enzyme products generated by the TF pathway and a weak inhibitor of the TF-FVIIa complex. TFPI and antithrombin have a synergistic effect and regulate kinetic thresholds so that the initiating TF stimulus must be of a significant proportion to introduce thrombin generation [13].

The effect of PC is evident only after thrombin is generated, due to the fact that PC is activated by the binding of thrombin to thromomodulin. APC binds competitively with FVIIIa and FVa, thus interfering with the production of the prothrombinase complex as well as the intrinsic Xase [13].

Alpha 2 macroglobulin (α2M) is a plasma proteinase inhibitor and inhibits endopeptidases of any class [19]. α2M acts as a minor inhibitor of thrombin in adults, as the level of α2M decreases with age [20]. Another minor player in the inhibition of thrombin is the heparin cofactor 2, which is a cofactor for heparin and dermatan sulfate and is also called "minor antithrombin" [21].

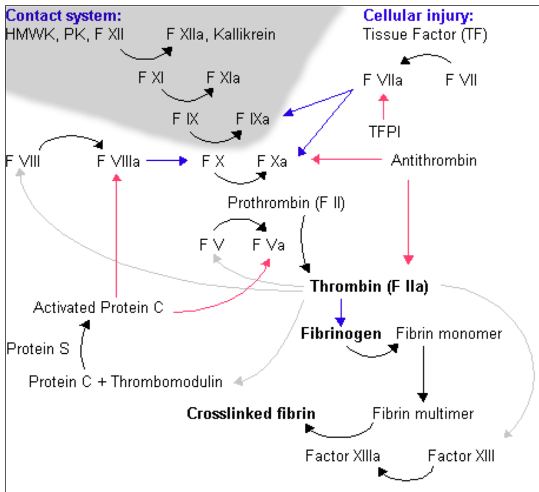

FIGURE 4. *A graphic presentation of the coagulation system illustrating the inhibition phase of thrombin generation (in white). (Source: http://en.wikipedia.org/wiki/Image:Coagulation_cascade.png. Image modified by Jonas Walldén.)*

2.3 Markers of thrombin generation

Since thrombin plays a central role in the coagulation cascade, many studies have focused on establishing a reliable and "operator-friendly" method to assess the generation of thrombin by measuring appropriate markers. Because the levels of thrombin generated during the initiation phase are very low, and typically below the detection level by the most commonly used substrates, this early phase of thrombin generation is often referred to as the "lag" phase. The rapid burst of thrombin production that occurs on the surface of the activated platelet during the propagation phase is called "maximum rate". Thrombin production slows following that burst and the concentration of free thrombin reaches a maximum thrombin concentration (peak). The time it takes to reach this maximum concentration (time to peak) provides information about the total length of the reaction. After this time point, the amount of free thrombin able to cleave fibrinogen declines due to inhibition and returns to baseline. The area under the curve (AUC), or endogenous thrombin potential (ETP), is a measure of the total amount of free thrombin present in the reaction from the point of initiation until the return to baseline [22].

2.4 What is "normal" thrombin generation?

Each individual's blood composition has a unique clotting profile. Both inherited (genetic) and acquired (environmental and therapeutically induced) haemostatic variations can lead to significant alterations in these profiles [23, 24]. It has been shown that the coagulation factors show remarkable interindividual variation, even among healthy subjects [24]. Several technologies have been developed to directly or indirectly measure thrombin generation profiles [23, 24, 25].

Brummel-Ziedens et al [25] used the controls from the Leiden Thrombophilia Study (LETS) to evaluate differences in thrombin generation among healthy individuals by utilising a numerical model. The results from this study demonstrated that there are healthy individuals who generate large quantities of thrombin fast (potentially prothrombotic) and others that generate lesser amounts of thrombin under a longer period of time (potentially protected from thrombosis). The authors showed that the subjects with higher thrombin production had also higher levels of procoagulant factors and lower levels of anticoagulants (antithrombin, TFPI). Women clotted faster and more than men and total thrombin increased with age. Individuals with body mass index (BMI)>26 had higher total and maximum levels of thrombin, as well as faster rates of thrombin generation and shorter lagtime.

In contrast to [25], Van Hylckama Vlieg et al [26], in another report from the LETS study, showed that there was no difference in ETP between men and women independently of the presence or not of deep vein thrombosis (DVT). In all subgroups, ETP levels increased slightly with age. ETP was higher in

women using oral contraceptives (OC) than in their counterparts who did not use OC [26], a finding confirmed also by Brummel-Ziedens et al [25].

Gatt et al showed that the levels of total and maximum thrombin in healthy individuals of both genders did not differ significantly. However, the lagtime and ttpeak in the presence of 5 pM TF was significantly different between females and males [27].

Devreese et al [28] compared thrombin generation markers in the plasma of healthy individuals (adults and children) measured by both a fluorogenic (Calibrated Automated Thrombogram, CAT®, at 2.5 pM TF) and a chromogenic method [Behring Coagulation System® (BCS®)]. They established different reference ranges for adults and children, with higher ETP and peak and shorter lagtime and ttpeak for adults than for children. No significant differences were observed between men and women (measured by CAT®, at 2.5 pM TF) [28].

Haidl et al [29] investigated the age-dependency of thrombin generation markers, as measured by CAT®, in the plasma of 121 children and 86 adults. The ETP of all children was significantly lower than that of adults. Younger children (0.5–6 years old) tended to have longer lagtime and ttpeak as well as lower peak and ETP values than the adults. Adults >35 years old had higher ETP and peak as well as shorter lagtime and ttpeak compared with adults <35 years. The authors hypothesized that those differences in thrombin generation markers are secondary to variations of other coagulation factors; namely lower prothrombin and higher antithrombin levels [30] in children, both of which affect thrombin generation [31].

2.5 The effect of coagulation parameters on thrombin generation

It has been shown [31] that antithrombin and prothrombin are the two factors that exert the strongest effect on thrombin generation markers. In [31], variations of factors V, VIII, IX and X (mean concentrations: 20 nmol/L, 0.7 nmol/L, 90 nmol/L, 170 nmol/L respectively) did not significantly affect the total concentration of thrombin. However, simultaneously halving of all those four factors decreased the amount of generated thrombin and prolonged the initiation phase. This is in agreement with the finding that individuals with coagulation factor levels up to 25–50% of the mean reference values do not exhibit any significant bleeding tendency [31].

Another study assessed the effects of all coagulation factors on thrombin generation measured by CAT® in coagulation factor-deficient plasma samples [32]. The plasma samples were spiked with different amounts (0–100%) of normal plasma to achieve the intended variety of concentrations for each factor. When thrombin generation was measured in plasma at low TF concentrations (1 pM), it was affected by all factors except factor XI. At a higher TF level (5 pM), thrombin generation was affected only by the factors of the extrinsic

pathway. ETP and peak correlated to FII concentration in a linear model. The influence of FX strongly depended on TF levels. In FIX- and FVIII- depleted plasma, ETP and peak were decreased to 60–70% and 25–30% of the normal values, respectively. Increasing fibrinogen levels increased thrombin generation. Decreasing fibrinogen levels decreased lagtime, but only at a low TF concentration (1 pM). On the contrary, increases in protein S prolonged lagtime, mostly at TF 1 pM. Decreased antithrombin concentrations lead to a marked increase in thrombin generation. PC had no effect, a result that was attributed to the absence of thrombomodulin in the assay [32].

Brummel-Ziedins et al [25] demonstrated that the only factor with an influence on the total amount of produced thrombin was FII. The maximum level of thrombin (peak) was affected by the coagulation factors in the following order: FII>FVII>TFPI>FV>antithrombin>FIX.

3 Thrombin generation assays

3.1 Why should thrombin generation assays be used instead of conventional assays?

Hemker et al [33] reported that the intra-individual variation of the ETP is c. 5% and the inter-individual variation is >15%. This means that 99% of all normal individuals have a thrombin generation between 55% and 145% of the mean value. Hemker and Dieri [34] illustrated those differences by referring to those "physiological" extremes as individuals with different thrombin generation profiles: "Mrs. High" and "Mr. Low". Mrs. High has a low, yet normal set of anticoagulant factors and a high, yet still normal, set of procoagulant factors, whereas Mr. Low has low procoagulant and high anticoagulant factors. This means that Mrs. High has the potential to produce up to 50% more thrombin than Mr. Low. Traditional coagulation assays cannot accurately express such differences.

Clotting times fail to assess the potentially significant normal variations of the haemostatic potential, even in the normal population. At the time when the clotting time is measured, only a tiny fraction of thrombin has been produced (<2%). The remaining thrombin is still to be converted from prothrombin, which means that the PC system is not yet activated and the plasma antithrombin cannot influence the process, rendering the clotting assays insensitive to the levels of those proteins [34]. Contrary to clotting times, thrombin generation is very sensitive to variations of prothrombin and antithrombin, even around the normal mean, as well as to the activity of the APC system [25, 32].

Additionally, the conditions of a thrombin generation experiment can be reproduced at almost any concentration of TF. The classical coagulation assays, activated partial thromboplastin time (aPTT) and prothrombin time (PT), are performed at either excessively high concentrations of TF (PT) or at an equally unphysiological absence of TF (aPTT). Measuring PT at lower TF concentrations is possible and has been practised but has never become a routine measurement, primarily because of difficulties to standardize and validate the method [34].

3.2 Techniques for the measurement of thrombin generation

Thrombin generation is far from being a novel technique. It has been used extensively as both a diagnostic and a research method [35].

The thrombin-antithrombin complex (TAT) and the quantification of the activation peptide released from prothrombin upon activation by factor Xa (prothrombin fragment 1+2, F1 + 2 fragment) measured by an immunological (ELISA) assay are surrogate markers for thrombin generation. Both methods have been used extensively and give an estimate of the amount of thrombin formed in blood at a given moment in time [36], but do not express the overall haemostatic potential.

Thromboelastography [37] and turbidity measurement (due to clot formation) [38], namely methods also used to assess clotting time, result in thrombogram-like curves. However, because the results depend on a dependent variable (turbidity), these methods reflect thrombin generation in an indirect way and their application is limited.

Two methods are commonly employed for the measurement of in situ thrombin generation: i) comprised manual sampling at timed intervals and determination of the concentration of thrombin in these samples, during clotting of blood or plasma [39, 40] and ii) a continuous method whereby total thrombin is detected by adding a suitable thrombin substrate to the clotting sample and monitoring the time course of appearance of the amidolytic split product [35]. The continuous method constantly records changes in optical density due to conversion of a slow-reacting chromogenic or fluorogenic substrate by thrombin generated in the sample [41]. The calculations assume that after a certain time there is no free thrombin in the sample [42].

The subsampling method [39] is a chromogenic method, which registers the changes of the thrombin-α2M complex by mixing timed aliquots from recalcified plasma with antithrombin+heparin (to neutralize free thrombin) and thereafter adding a chromogenic substrate. This method allows even for the effects of anticoagulants to be registered. However, the protocol is complicated and not "operator-friendly", as the amount of the thrombin-α2M complex has to be subsequently removed during the calculation of the total thrombin generation. Additionally, thrombin generation, measured as the rate of conversion of chromogenic substrate, is calculated directly from the recalcified plasma reaction mixture and thus does not correspond to the actual amount of generated thrombin (i.e. thrombin minus the thrombin-α2M complex) [42].

In most continuous chromogenic thrombin generation assays, the visual reproduction of the thrombin generation (i.e. the thrombin generation curve) is dependent on factors other than the rate of thrombin generation. For example, the optical signal is dependent on the decreasing rate of the substrate. To overcome this, excess substrate is used, which forms a reversible complex with thrombin, thus protecting it from inactivation. Thrombin inhibition affects the natural procedure and requires the addition of extra antithrombin for neutralization and correct measurement of AUC. This is a common problem in all continuous methods [35]. The clotting plasma sample leads to changes in opti-

cal density. In the chromogenic methods, fibrinogen and platelets are removed from the biological sample prior to measurement [35].

In the fluorogenic assays, the optical signal is not linearly dependent on the concentration of fluorescent product, due to the so-called 'inner filter effect', namely the ability of the fluorescent molecules to absorb the light from other product molecules. By increasing substrate concentrations as required to limit the effect of substrate consumption, the inner filter effect automatically increases [33].

Devreese et al [28] compared a chromogenic method (as used in the fully automated Behring Coagulation System, BCS®) with a fluorogenic method (CAT®) for the measurement of thrombin generation when using various TF concentrations. In the presence of high TF concentrations, as used in the BCS® method (300 pM), thrombin generation is affected merely by the factors of the extrinsic pathway. When low concentrations of TF are employed in the BCS®, both the sensitivity and reproducibility of the method were lower, which was attributed by the authors to the presence of a neutralizing FVII antibody. Thrombin generation is lower when measured by BCS®, most likely because of low phospholipid concentration.

3.3 The Calibrated Automated Thrombogram® (CAT®)

The CAT® was developed by Hemker et al to assess the overall haemostatic potential [33, 43]. As mentioned above (3.2), thrombin generation assays are long-established reliable methods of validation of the clotting system. The subsampling method that was previously employed is time- and material-consuming, producing one thrombin generation curve per hour and not "operator-friendly". The CAT® method, on the other hand, enables c. 100 thrombogram curves per man-hour to be obtained (Image 1).

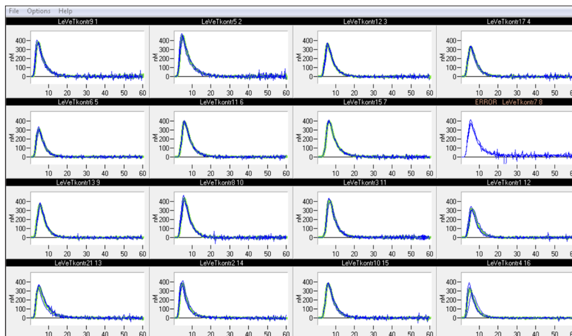

IMAGE 1. *Thrombin generation curves obtained by CAT® (samples from the controls used in Paper IV, in PPP, 5 pM TF and 4 µM phospholipids). The images were obtained from the author's archive.*

3.3.1 Outline of the method

In a 96-well round bottom plate, 20 µL of pre-warmed trigger solution (Platelet Rich Plasma, PRP or Platelet Poor Plasma, PPP reagent) is added to one well and 20 µL of pre-warmed calibrator (Thrombin Calibrator, α2M-thrombin complex) to another; 80 µL of plasma (PRP or PPP) is added to both wells. Ca^{2+} is added together with the fluorogenic substrate (FluoSubstrate) immediately prior to the beginning of the measurement (zero time) (Fluca Solution). The readings are done in a microtiter plate fluorometer (Fluoroscan Ascent, Thermolabsystems, Helsinki, Finland), at 37°C (Image 2).

When the experiment is started, a dispenser squirts 20 µL of FluCa solution in each well at zero time. During the measurement, the program compares the readings from the thrombin generation and the calibrator wells, calculates thrombin concentration and displays the thrombin concentration in time. On the computer screen connected to the instrument appears a picture of the signal from the calibrator and the calculated thrombin as a curve. The displayed thrombin concentration is a preliminary value because the α2M-thrombin complex builds up from the thrombin continuously generating in the experiment and the α2M normally present in any plasma. That is a common feature of both the continuous and the subsampling method, in which thrombin is estimated by its amidolytic activity on a small signal-substrate (see 3.2). This is corrected by the software program (Thrombinoscope, Synapse BV, Maastricht, The Netherlands), which enables the identification of the (sets of) wells and determines the duration of the experiment and the sampling rate (usually 4/min). Thrombin generation can be continuously measured up to 60 minutes [35, 44].

IMAGE 2. *The instrument, reagents (PPP reagent, thrombin calibrator, FluCa) and plates used. (Photo by professor Tomas L Lindahl.)*

The presence of the thrombin calibrator allows for correction of the inner filter effect, substrate consumption and the colour differences between plasmas from different donors. The manufacturers advise that each experiment (clotting plasma and calibrator) is performed at least in duplicate [35].

It has been suggested that the presence of the artificial thrombin substrate could act as a competitive thrombin inhibitor and thus interfere with the thrombin generation mechanism [45]. Hemker and de Smedt [46] have shown, however, that the extent and the time course of prothrombin conversion as well as thrombin-generation inhibition are minimally disturbed by the presence of the substrate at the usual concentration.

The reagents used in the studies included in this thesis are: Thrombin Calibrator: 20 mM Hepes, 140 mM NaCl, 5 g/L bovine serum albumin (BSA), pH 7.35, with 0.02% sodium azide as a preservative; FluCa solution: Buffer with 60 mg/mL of BSA, 0.1 M $CaCl_2$ and 2.5 mM of Z-Gly-Gly-Arg-AMC (Bachem, Switzerland); trigger solution for PPP that after addition of 20 µL FluCa solution contains 5 pM TF and 4 µM phospholipids (final reaction mixture) and were obtained from Thrombinoscope BV, The Netherlands (Image 2) [35].

In images 3A and 3B thrombin generation curves in the plasma of a patient with factor XIII (FXIII) deficiency (FXIII 0.29 kIU/L at the time of the blood sampling, reference range 0.7–1.4 kIU/L) are shown. Thrombin generation was measured both in PPP (3A) and PRP (3B, platelet count adjusted to 200 × 10^9/L). In accordance with the available data, i.e. that FXIII deficiency cannot be diagnosed by thrombin generation assays [33], thrombin generation both in PPP and PRP is normal.

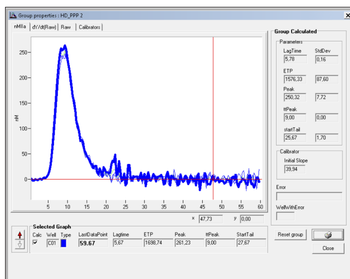

IMAGE 3A. *Thrombin generation measured in PPP from a patient with FXIII deficiency.*

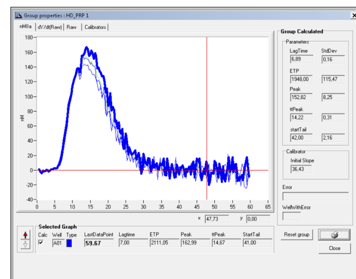

IMAGE 3B. *Thrombin generation measured in PRP from a patient with FXIII deficiency.*

Although the method is “operator-friendly” and easy to use, caution is imperative when performing the experiments. Minor mistakes, such as the presence of small air bubbles in the wells, but even wrong pipetting, errors during the preparation of the samples, delay in starting the measurement (PPP and

Thrombin Calibrator reagents, for example, are stable only one hour at room temperature following reconstitution) might disturb the measurements, resulting in erroneous readings. In images 4–9 some of the most common errors are illustrated; in image 9 the results cannot be used, despite the fact that the instrument did not interpret it as “error”. This shows the importance of careful and critical review of the data prior to interpretation. In all cases, the blood samples for the measurement were drawn from healthy controls without anti-haemostatic medication (PPP, 5 pM TF, 4 µM phospholipids). The images were obtained from the author’s archive.

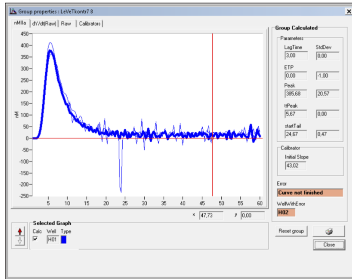

IMAGE 4. *Error: Curve not finished.*

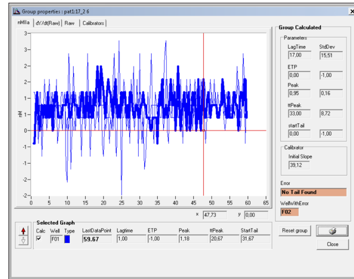

IMAGE 5. *Error: No tail found.*

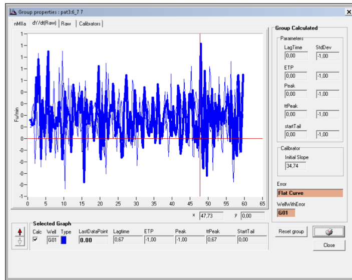

IMAGE 6. *Error: Flat curve.*

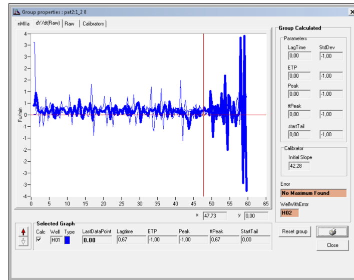

IMAGE 7. *Error: No maximum found.*

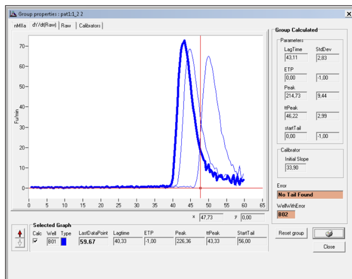

IMAGE 8. *Error: No tail found.*

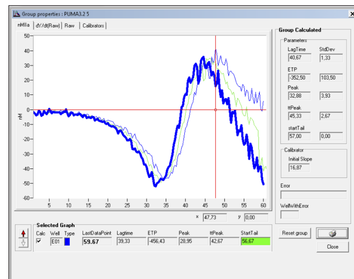

IMAGE 9. *Negative ETP, which makes the results unusable.*

3.3.2 Thrombin generation markers measured by CAT®

The markers measured by CAT® are: lagtime (clotting time, the moment at which thrombin generation begins), endogenous thrombin potential (ETP, AUC, the total amount of thrombin generated, in nanomolar*minute), peak height (maximum thrombin concentration, in nanomolar thrombin), ttpeak (time to reach maximum thrombin concentration) and starttail (time at which thrombin generation has come to an end, in minutes) [Image 10 (10A, 10B, 10C)]. Starttail, however, is an instrument/software-generated parameter and is not always biologically relevant [44]. Starttail was not studied in the papers included in this thesis, with the exception of Paper I.

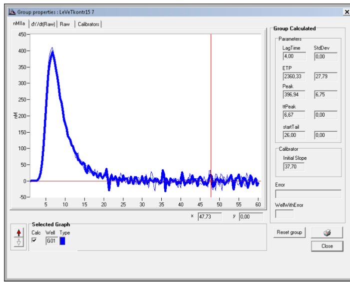

IMAGE 10A.

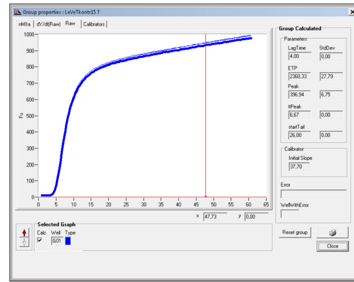

IMAGE 10B.

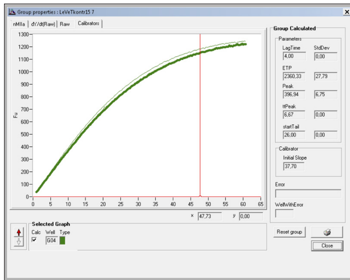

IMAGE 10C.

IMAGE 10. *Thrombin generation curve (10A) as well as raw data (10B) and calibrator data (10C) in PPP from a healthy individual (5 pM TF and 4 μM phospholipids). The images were obtained from the author's archive.*

The levels of thrombin generated during the initiation state are below the level of detection by most commonly used substrates; this early phase of the thrombin generation curve is often referred to as the lag phase. The rapid burst of thrombin generation that occurs on the surface of the activated platelets during the propagation stage is called the maximum rate. Thrombin production slows following this maximum burst and the concentration of free (uninhibited) thrombin reaches a peak. The time to reach this peak provides informa-

tion about the total length of the strongly procoagulant phase of the reaction. Following the time at which the peak level of free thrombin is reached, the concentration of free thrombin able to cleave fibrinogen declines due to physiological inhibition and returns to baseline. The area under the curve, or ETP, is a measure of the amount of free thrombin present in the reaction from the point of initiation until the return to baseline [22].

3.3.3 Preanalytical variables that might affect thrombin generation assays

The preanalytical phase extends from the collection of the sample to the time of the testing and it is the phase in which >60% of laboratory errors occur [47]. As the haemostatic system is activated during the preanalytical phase, coagulation tests are susceptible to errors secondary to inadequate sample quality [48].

One of the major issues that have been discussed since the introduction of thrombin generation assays, and particularly the CAT® assay, is the standardization of the method. Since no international reference ranges have been defined and the preanalytical conditions (method of blood collection, centrifugation, method of storage etc.) vary significantly in each laboratory, it is complicated to compare the results from different studies. Similarly designed studies on similar cohorts have produced different results (see Papers I and II), and part of the explanation can be found in the presence of different conditions prior to analysis. Some authors have concentrated on studying the effect of different preanalytical conditions on the result of thrombin generation, rather than thrombin generation in different cohorts, as in this thesis, as the blood sampling and handling is important for the credibility of the final results.

In thrombin generation assays that use a low TF concentration as a trigger, contact activation is a major issue. This problem is solved by sampling the blood into an anticoagulant that contains corn trypsin inhibitor (CTI), an efficient and specific inhibitor of the activated factor XII (FXIIa). The extent of the contact activation greatly depends on the concentration of TF used: for concentrations below 15 pM, FXIIa-driven thrombin generation can equal or even exceed that due to TF (extrinsic) pathway. At concentrations >15 pM this effect is greatly diminished [49]. Luddington et al suggested that blood should be collected in tubes containing CTI as addition of CTI after plasma separation was not sufficient even at TF concentrations 5 pM TF [49]. Van Veen et al [50] showed that CTI addition is unnecessary when the trigger contains 5 pM TF. In a similar fashion, Spronk et al [51] showed that neither addition of CTI after plasma separation nor collection of whole blood in tubes prefilled with CTI was necessary when the thrombin generation measurement is performed at the presence of TF concentration >1 pM.

Gatt et al [27] measured thrombin generation at the presence of 5 pM TF and 4 μM phospholipids in the plasma of patients with atrial fibrillation treated with warfarin using different sample tubes and noted significant differences in

thrombin generation parameters when different tubes were used. Thrombin generation was also measured in the blood samples of six individuals on warfarin treatment, sampled in Vacutainer® tubes both with and without CTI. No significant difference was found. Some samples transported via the PTS (pneumatic tube system) were visibly haemolysed but no significant differences were noted between the results obtained from the samples carried by hand and those sent via the PTS. The authors also reported that thrombin generation measurements in frozen-thawed PPP and fresh PPP gave the same results [27].

Duchemin et al studied the influence of TF and phospholipids on thrombin generation. At a low TF concentration, all factors except factor XI influenced thrombin generation. At a high TF concentration, only the factors of the extrinsic pathway exerted an influence. ETP and peak increased with increased TF, whereas lagtime exhibited a decreasing tendency [32].

Thrombin generation measured by means of CAT® was estimated in 20 healthy individuals in fresh PRP and ft (frozen-thawed) PRP [52]. Ft-PRP samples exhibited higher ETP and peak, as well as shorter ttpeak. This was probably due to the fact that the freeze-thaw cycles damage the platelet membrane, which, in its turn, leads to abnormal exposure of the procoagulant phospholipids [53].

Recently, Loeffen et al performed a structured evaluation of the effect of preanalytical variables on the results obtained from the CAT® when thrombin generation was measured in the presence of 0, 1 and 5 pM TF [54]. When comparing three collection devices (intravenous catheters, butterfly needles and straight needle), it was shown that when thrombin generation was measured in the presence of 5 pM TF, no significant difference was found in endogenous thrombin potential (ETP) and peak height between the three collection devices. However, significant differences were shown for both 0 and 1 pm TF. At the same time, haemolysis was more common when samples were drawn through a butterfly device or an intravenous catheter, compared with a straight needle, although other studies have found no difference between the butterfly device and the conventional straight needle [55, 56]. Blood was collected in seven different collection devices, one of which had CTI added to it. It was shown, that when thrombin generation was measured in the presence of 0 and 1 pM TF, ETP and peak exhibited considerable variations depending on the presence or absence of CTI; no such variation were shown when thrombin generation was measured in the presence of 5 pM TF, however. ETP was 0 when measured in the presence of 0 pM TF in the tube containing CTI, indicating that CTI inhibits contact activation completely.

Concerning centrifugation, it was shown that, regardless of whether the blood samples were collected in Vacutainer glass tubes or Monovette plastic tubes, when thrombin generation was measured in the presence of 5 pM TF, single or double centrifugation did not lead to considerable differences in the results of peak and ETP. Likewise, the stability of both whole blood, when it was incu-

bated for 6 h at 4°C, room temperature or 37°C, and plasma, when incubated at the same temperatures for variable time, did not affect the results when thrombin generation was measured in the presence of 5 pM TF. However, thrombin generation results, as expressed by ETP and peak, were affected by these conditions when the measurements were performed in the presence of 0 and 1 pM TF.

The authors [54] recommend double centrifugation, addition of CTI to the sample and immediate analysis of plasma following thawing. However, as shown above, preanalytical variables affect thrombin generation when the samples are analyzed only in the presence of no or low concentrations of TF.

The fact that thrombin generation measured in the presence of 5 pM TF does not depend on single or double centrifugation is illustrated in this thesis as well: in both Papers I and II (same cohort), there were no statistically significant differences between the ratios [thrombin generation marker measured following single centrifugation]/[thrombin generation marker measured following double centrifugation] for patients with and without the factor V Leiden (FV Leiden) mutation.

In Paper IV, thrombin generation was measured in plasma that was centrifuged once prior to freezing and once immediately following thawing. As the results of the CAT® assay at the presence of 5 pM TF are not affected by incubation of the plasma following thawing, it is not probable that this handling affected the results. Likewise, CTI was not used in any of the samples upon collection. As all of the measurements were performed in PPP under standard conditions, i.e. 4 µM phospholipids and 5 pM TF, contact activation was not a major issue [50, 51].

The effect of platelets was not an important parameter in any of the cohorts included in this thesis. Because of that, as well as for practical reasons, PPP was used in all the experiments.

3.3.4 Applications of CAT®

As reflected in this thesis, thrombin generation assays can be used as a diagnostic or research tool in a variety of clinical conditions. In Part II, data are presented on the utility of thrombin generation assays in patients with thrombophilia, as a risk marker for recurrent venous thromboembolism (VTE), during the menstrual cycle, in patients with liver diseases and in patients with mild FVII deficiency.

Thrombin generation assays can be used both for the diagnosis of factor deficiencies (except for FXIII deficiency, as illustrated even in 3.3.1) [35] and for diagnosis of hypercoagulability [57].

Thrombin generation assays have gained increasing importance in diagnosis and follow-up of management in haemophilia [58]. Thrombin generation assays can be used to distinguish between different bleeding phenotypes in pa-

tients with mild/moderate hemophilia A [59] or to predict the response when bypassing haemostatic agents are administered to patients who have haemophilia with inhibitors [60].

Additionally, it has been shown that global haemostatic methods such as thrombin generation assays can be useful in the perioperative management of patients with a bleeding diathesis, both for monitoring but even for evaluating the treatment effect following administration of blood products and antihaemostatic agents [36], as the commonly used thromboelastography follows changes mainly in fibrinogen and offers little information on the fluctuations of thrombin formation.

Thrombin generation assays have also been used in the research of cardiovascular diseases, although their use is much more limited than in the respective field of venous thrombosis due to differences in the pathogenesis of those diseases [36]. Thrombin generation is elevated following an acute myocardial infarction [61, 62], but it is low, rather than high thrombin generation that appears to be associated with recurrent episodes [62]. However, acute inflammation and vessel injury at the time of an acute myocardial infarction might influence the results.

Another application of thrombin generation assays is found in the monitoring of anticoagulants. It is well known that treatment with coumarins affects thrombin generation [27], but in that case monitoring occurs by measuring prothrombin time-international normalized ratio (PT-INR). However, monitoring the effect of the new oral anticoagulants (NOAC), which inhibit thrombin or FXa is not as well standardized [63]. NOAC have rather predictable pharmacokinetics and pharmacodynamics. There are, however, some instances in which monitoring and determination of the effect of the NOAC is desirable, such as in the event of an adverse event; global assays such as thrombin generation might have a role in this context.

Part II

Thrombin generation in specific cohorts

1. **Thrombin generation and thrombophilia. Paper I.**
2. **Thrombin generation and risk for recurrent venous thrombosis. Paper II.**
3. **Thrombin generation during the menstrual cycle. Paper III.**
4. **Thrombin generation in patients with liver disease. Paper IV.**
5. **Thrombin generation in patients with mild factor VII deficiency. Paper V.**
6. **Conclusion and future perspectives**
7. **References**
8. **Acknowledgements**

1 Thrombin generation and thrombophilia. Paper I.

1.1 Introduction

1.1.1 Factor V Leiden and prothrombin G20210A mutation: the most common causes of hereditary thrombophilia

Hereditary resistance to the inhibitory effect of APC in plasma is most commonly secondary to the presence of FV Leiden, i.e. a mutation in FV, and associated with an increased risk of venous thromboembolism [64, 65]. This single nucleotide polymorphism (Arg506Glu) in the APC cleavage site of activated FV renders the FV molecule less susceptible to inactivation by APC [64], and is the leading cause of inherited thrombophilia, affecting up to 10% of the Caucasian population [66]. APC resistance in the absence of FV Leiden, i.e. acquired APC resistance, is predominantly observed in women and often associated with changes in hormonal status occurring during pregnancy [67], the use of oral contraceptives (OC) [68] or the use of hormone replacement therapy [69]. Heterozygosis for FV Leiden has been associated with a 5–10-fold increased risk for a first-time thrombosis whereas homozygosis increases the risk for thrombosis about 50–100-fold [70, 71, 72]. Other studies, however, report lower risk: 2–5 times higher risk for heterozygous carriers of FV Leiden and 20–50 times higher for homozygous carriers [73].

A genetic variation in the 3'-untranslated region of the prothrombin gene, a G3A transition at nucleotide position 20210 (prothrombin G20210A mutation, PTG20210A), has been associated with an increased risk of venous thrombosis [74, 75]. The relative risk (RR) for venous thrombosis among carriers of this variant is 4.2, compared with a population without this mutation [75]. The prevalence of the PTG20210A mutation in the general population has been estimated to be about 2%, with considerable geographic variations [76], rendering the PTG20210A mutation one of the most prominent inherited thrombophilic factors.

1.1.2 Thrombin generation in patients with factor V Leiden and the prothrombin G20210A mutation

Lincz et al [77] measured thrombin generation in the presence of Protac®, a commercial snake venom extract, which is a specific activator of PC. The authors showed that the ETP ratio [ETP measured in the presence of Protac®]/[ETP measured in the absence of Protac®] was significantly higher in FV Leiden heterozygotes than in controls. Within the FV Leiden group, patients with a history of thrombosis had higher, but statistically not significant, ETP ratios

compared with those without [77]. Hron et al, however, refuted the notion that thrombin generation is enhanced in individuals who are carriers of the FV Leiden mutation. Thrombin generation markers were measured in the plasma of patients who suffered a thrombotic episode in a median of 13 months after discontinuation of anticoagulant therapy. No difference in peak thrombin generation was seen between patients with and without FV Leiden [78].

Kyrle et al showed that the ETP was significantly higher in patients who were heterozygous for the PTG20210A mutation compared with controls, and excessively high levels were found in two homozygous carriers that were included in the study [79]. Hron et al, in a report from the AUREC study, established that in patients with VTE, carriers of the PTG20210A mutation had significantly higher peak thrombin generation than patients without the mutation [78].

1.2 Paper I

1.2.1 Aim

The aim of this study was to evaluate differences in thrombin generation and D-dimer values between carriers and non-carriers of FV Leiden or the PTG20210A mutation. At the time of the blood sampling, all subjects were suspected of having VTE (DVT or pulmonary embolism, PE). The effects of confounders such as gender and the presence of VTE were evaluated.

As no thrombomodulin or Protac® was used in the assay, it was not expected that the resistance to APC observed in individuals with FV Leiden would affect the results. Thus, any observed difference would have to be secondary to other mechanisms that could be influential on thrombin generation in patients with FV Leiden. Such a finding could be a part of the explanation behind the differences in the phenotypes of patients with FV Leiden, i.e. that most of them do not experience any thromboembolic episodes whereas others suffer recurrent thrombotic episodes [77].

1.2.2 Patients and controls

The study cohort consisted of 98 patients who were carriers of the FV Leiden (87 heterozygotes and 11 homozygotes) and 15 patients who were heterozygous carriers for the PTG20210A mutation, as well as 98 patients without inherited thrombophilia who were age- and gender-matched to the cohort with FV Leiden (denoted as FV Leiden controls) and 15 patients without inherited thrombophilia who were age- and gender-matched to the cohort with the PTG20210A mutation (denoted as PTG20201A controls).

The cohort for Paper I was part of a larger cohort from the Linköping Study on Thrombosis (LIST), which is described in detail in the corresponding section of Paper I.

1.2.3 Blood sampling and handling

Immediately after inclusion in the study, blood samples were drawn and collected in citrated tubes containing 1/10 volume 0.13 mol/L sodium citrate. The samples were subsequently centrifuged for 15 min in 2500 g. The supernatant was then collected 0.5 cm from the blood surface and pooled respectively for each patient. The pooled plasma was divided into aliquots and stored at -70°C.

1.2.4 Measurement of thrombin generation by Calibrated Automated Thrombogram®

See corresponding section in Paper I for details. The final mixture of PPP reagent (trigger) and PPP used in the assay contained 5 pM TF and 4 µM phospholipids.

All thrombin generation measurements were performed by the author.

Of the 226 patients (with and without thrombophilia) included in the study, thrombin generation results were usable for 198. Ten patients with FV Leiden and 7 of the FV Leiden controls were excluded due to technical errors during the measurement of thrombin generation (see 3.3.1 for some common errors). Three FV Leiden controls, 5 heterozygotes and 1 homozygote for FV Leiden were excluded due to concomitant treatment with warfarin at the time of the blood sampling.

All in all, 82 patients with FV Leiden and 88 FV Leiden controls were included in the statistical analysis for thrombin generation.

One patient with the PTG20210A mutation and PTG20210A control were excluded due to treatment with warfarin at the time of the inclusion.

1.2.5 Statistical analysis

All the results were analysed by ANOVA’s Estimate Model (SYSTAT 11). Due to the non-normal distribution of the data, all the numerical variables were expressed as logarithms. The statistical significance was evaluated via Tukey’s HSD multiple comparisons test ($p<0.05$).

The methods for identifying FV Leiden mutation, PTG20201A mutation, diagnosis of DVT and PE and analysis of D-dimer are described in the corresponding section in Paper I.

1.3 Results

1.3.1 Thrombin generation markers and D-dimer in patients with factor V Leiden and factor V Leiden controls

No differences were observed for the thrombin generation markers between the FV Leiden (both heterozygous and homozygous carriers) and the FV Leiden controls.

Neither age nor gender had any significant effect on those results.

The D-dimer values were significantly higher in the group with FV Leiden (heterozygotes, p=0.001 and homozygotes, p=0.014) than in the FV Leiden controls. This difference was secondary to the higher number of VTE in the FV Leiden group compared to controls (41 vs. 22 in the FV Leiden control group, multivariate analysis). Neither age nor gender had any effect on the results.

1.3.2 Thrombin generation markers and D-dimer in patients with prothrombin G20210A mutation and prothrombin G20210A controls

No differences were observed for the thrombin generation markers and the D-dimer between the patients with PTG20210A mutation (both heterozygous and homozygous carriers) and the PTG20210A controls.

Due to the small number of subjects in this cohort, no further multivariate analysis was performed.

1.3.3 Thrombin generation in patients with factor V Leiden and factor V Leiden controls with and without thrombosis upon inclusion

The patients with thrombosis upon inclusion (both patients with FV Leiden and FV Leiden controls) had significantly longer lagtime (p=0.007, 5±3.9 min vs. 4.3±2.9 min) and slightly, but not statistically significant longer ttpeak (p=0.061, 8.6±4.7 min vs. 7.4±2.9 min) than the rest of the subjects (FV Leiden and FV Leiden controls) without thrombosis upon inclusion (Table 1).

When a subgroup analysis was performed, it was shown that both FV Leiden controls and FV Leiden heterozygous patients with confirmed thrombosis upon inclusion had significantly longer lagtime (p=0.007 and p=0.017, respectively) compared with their respective groups without thrombosis (i.e. FV Leiden controls without thrombosis and FV Leiden heterozygous patients with thrombosis, respectively). The FV Leiden controls with thrombosis had additionally slightly longer ttpeak than the FV Leiden controls without thrombosis (p=0.061, 7.4±2.9 min vs. 7±1.5 min) (Table1).

1.4 Discussion

The protocol used in this study rendering the thrombin generation assay insensitive to APC for patients with the FV Leiden mutation by not adding thrombomodulin. The reason for that was the intention to examine other potential factors that could influence thrombin generation in patients with FV Leiden besides APC. The results indicate that if no thrombomodulin is used, thrombin generation does not differ between patients with and without FV Leiden, independently of the presence of an acute thrombosis at the time of the blood sampling. This could be indicative of the fact that thrombomodulin or other activators of PC, such as Protac®, are instrumental when evaluating thrombin generation in cohorts consisting merely of patients with FV Leiden, even if the majority of patients are heterozygous for the mutation.

It was shown that neither gender nor age had any effect on thrombin generation in this cohort. It has previously been suggested [29] that age could affect thrombin generation, but there is not an established praxis to correct for age and gender when studying thrombin generation in adult cohorts (personal communication from professor HC Hemker).

The controls for the patients with the PTG20210A mutation had experienced more thrombotic episodes than the patients with the mutation. This was not intentional, as the groups were designed to be age- and gender-matched, but not diagnosis-matched. It could, however, be a source of bias. This, combined with the fact that the number in both patient and control groups was small (n=15), discourages from drawing any conclusions from those results.

At the time of inclusion in the study, it was unknown which of the subjects had a thrombosis. As this study was designed to evaluate the effect of thrombophilia with thrombosis as a confounder, it was not deemed necessary to match the groups for diagnosis. The presence of thrombosis was, however, evaluated as a confounder by a multivariate analysis (ANOVA), showing no effect in this context.

The blood was centrifuged only once. However, in order to ensure that there was not any significant residual platelet activity that could have affected the results, 20 samples were randomly picked (8 from patients that were carriers of for the FV Leiden and 2 from patients who were carriers of the PTG20210A mutation and 10 from patients without this mutation), in a similar fashion as described in Paper II, and centrifuged one more time (at 2500 g, 15 min). Thrombin generation was subsequently analyzed again and there were no statistically significant differences (Mann-Whitney *U* test) between the results from the plasma centrifuged once and the plasma centrifuged twice [patients with thrombophilia; lagtime (min): 3.3 (2.6–5.7) vs. 3.2 (2.3–6.1) p=0.85, ETP (nM*min): 1874 (1336–1973) vs. 1838 (1322–2120) p=0.821, peak (nM): 317 (272–490) vs. 341 (229–400) p=0.597, ttpeak (min): 6.5 (5.1–9.5) vs. 5.8 (4.3–8.9) p=0.272, starttail (min): 21.8 (18.7–25.3) vs. 22.3 (19.3–26.7) p=0.116. Controls; lagtime (min): 3.6 (2.7–4.8) vs. 3.4 (2.3–6) p=0.82, ETP (nM*min):

1662 (1270–2441) vs. 1657 (1496–2307) p=0.734, peak (nM): 317 (231–380) vs. 339 (219–406) p=0.623, ttpeak (min): 6.5 (4.8–8.1) vs. 6.1 (4.8–9.2) p=0.545, starttail (min): 22 (18.7–28.3) vs. 21.7 (18–30) p=0.677].

The patients who were heterozygous for FV Leiden and the FV Leiden controls with thrombosis upon inclusion had longer lagtime (clotting time) than did the patients without thrombosis. This was not an effect of FV Leiden, as the same results were obtained from the controls with thrombosis and without thrombophilia. This finding was rather surprising, as ETP (total amount of produced thrombin) is the parameter that has been mainly used as a marker for hypercoagulability and risk of thrombosis [26, 80]. Interestingly, one of the main findings in Paper II (see corresponding chapter) was that the patients from the LIST cohort who had a thrombosis at inclusion and did not experience recurrences during a seven-year follow-up had prolonged lagtime at the time of the first thromboembolic episode. In the majority of the published studies in the field, blood sampling takes place later during the course of the thromboembolic disease and not at the time of the acute episode. The fact that prolonged clotting time, which suggests a tendency towards hypocoagulability, is linked to acute thrombosis and the risk for later recurrences is intriguing. Hyperfibrinolysis and inflammation might provide an explanation, as the close relationship between inflammation and coagulation, and especially the role of thrombin, is well established [81]. Unfortunately, due to the limited amount of available plasma, it was not possible to measure biomarkers of inflammation. Therefore, a direct conclusion of the significance of prolonged lagtime in this context cannot be drawn, as factors other than the alterations in the coagulation system secondary to thrombosis might have played a role.

As the use of phospholipids in concentrations greater than 1.5 μM has been shown to minimize the effect of residual platelet activity [82], the results obtained from frozen-thawed samples used in this study (both the results presented and the results for the ratios described above) were not considered to have been significantly affected by residual platelet activity.

1.5 Conclusion

In conclusion, thrombomodulin is necessary to thoroughly evaluate thrombin generation in patients with FV Leiden. Even in the presence of acute thrombosis and thus acute inflammation and activation of the coagulation cascade it does not appear that patients with FV Leiden have higher haemostatic potential than do controls in the absence of thrombomodulin. Larger studies evaluating the effect of inflammation on thrombin generation markers, especially clotting time, could prove useful in providing an explanation for the presence of prolonged lagtime at the time of a hypercoagulable event such as an acute thrombosis.

1.6 Tables

	lagtime (min)	ETP (nM*min)	peak (nM)	ttpeak (min)	D-dimer (mg/L)
all with VTE	5±3.9	1619±510	278±116	8.6±4.7	0.8±2.2
all without VTE	3.8±1.6	1676±306	293±67	7.1±2.9	0.3±0.4
FV Leiden	4.4±3	1631±414	288±96	8±4.5	1.5±4.3
FV Leiden controls	4±1.9	1756±426	296±73	7.1±1.9	0.7
FV Leiden with VTE	5±3.9	1619±510	278±116	8.6±4.7	2.9±6.3
FV Leiden controls with VTE	4.3±2.9	2009±650	329±95	7.4±2.9	2.4±4.1
FV Leiden without VTE	3.6±1.7	1698±311	306±74	7.2±4.3	0.4±0.5
FV Leiden controls without VTE	3.9±1.5	1662±305	285±62	7±.5	0.3±0.3
PTG20210A	4.3±3.2	1954±434	323±77	9.4±7.7	2.1±4
PTG20210A controls	4.4±3.3	1742±471	319±80	7.1±3.4	1.7±4

TABLE 1. *Thrombin generation markers and D-dimer values for the patients with thrombophilia and their respective controls (values expressed as mean±SD).*

2 Thrombin generation and risk for recurrent venous thrombosis. Paper II.

2.1 Introduction

2.1.1 Venous thromboembolism as a chronic disease

VTE is considered as a chronic, rather than an acute, disease due to the high risk of recurrence, especially following an unprovoked thrombosis [83, 84]. The risk of recurrence varies from 23.2% within the first four years following the initial thrombotic episode [85] to up to 30% within 10 years after the initial episode [86].

Factors such as male sex, inherited thrombophilia, the presence of antiphospholipid antibodies, familial thrombosis, age <50 years and abnormal D-dimer following discontinuation of anticoagulant therapy are recognized as risk factors for recurrences [83, 84, 87, 88]. Malignancy and the use of combined oral contraceptives are among the transient risk factors for both a first and a recurrent episode of venous thromboembolism [83, 89, 90].

One of the main problems faced by the clinician is deciding the optimal duration of treatment with anticoagulants following a thrombosis. As this decision is based mainly on the risk for recurrence and therefore the relevant risk factors, designing an algorithm that accurately predicts this risk is of great importance for the treatment of the patient.

2.1.2 Thrombin generation as marker for recurrence risk

Thrombin generation has been studied as a marker for recurrence risk following a first thrombotic episode in studies of diverse design. The results of these studies, even under the same conditions, are rather conflicting.

Sonnevi et al showed that thrombin generation measured in the presence of APC following discontinuation of anticoagulants can be used as a risk factor for recurrence in women aged 18–65 years who have experienced a thrombotic episode [91]. Besser et al [92] reported that high ETP measured in the absence of thrombomodulin could indicate the patients at risk of a recurrence especially following an unprovoked episode. Those results were confirmed by Eichinger et al [80].

ETP measured in the presence of thrombomodulin has been associated with increased risk of a first, but not a recurrent episode of VTE [26]. However, when Tripodi et al [93] measured thrombin generation in the presence of thrombomodulin under different conditions than in [26], using lower amounts of tissue factor and phospholipids (1 vs. 15 pM tissue factor and 1 vs. 4 μM phospholipids), it was shown that increased ETP and peak were indeed risk factors for recurrence. Peak thrombin concentration lower than 400 nM following discontinuation of anticoagulants has been associated with low recurrence risk [78].

2.2 Paper II

2.2.1 Aim

The aim of this paper was to study whether variations in thrombin generation profiles, measured at the time of the initial thromboembolic episode, can be used as early markers for prediction of the risk of recurrence during a seven-year follow-up. The implications of such findings could include prolonged treatment with anticoagulants for patients perceived as being at high risk of a recurrence.

Additionally, the thrombin generation profiles at the time of the initial episode are compared with thrombin generation profiles obtained about 1–2 months following discontinuation of the treatment with anticoagulants.

2.2.2 Patients

The cohort of this study was part of the larger cohort from the Linköping Study on Thrombosis (LIST). The present cohort includes only the patients that were diagnosed with thrombosis at the time of the inclusion. Of the 158 patients with thrombosis at inclusion, thrombin generation profiles were obtained for 115. The missing profiles were mainly the result of technical errors during the analysis or the preparation of the blood sample or not enough plasma available for analysis.

No recurrence rates are mentioned, as the cohort studied in Paper II is only a part of the patients with thrombosis on inclusion.

The main characteristics of the patients included (n=115) are shown in Table 1.

Additionally, thrombin generation was analysed 1–3 months following discontinuation of anticoagulants for 58 patients.

2.2.3 Blood sampling and handling

Immediately after inclusion in the study, blood samples were drawn and collected in citrated tubes containing 1/10 volume 0.13 mol/L sodium citrate. The samples were subsequently centrifuged for 15 min in 2500 g. The super-

natant was then collected 0.5 cm from the blood surface and pooled respectively for each patient. The pooled plasma was divided into aliquots and stored at -70°C.

The same procedure was followed for the samples collected following discontinuation of anticoagulants.

2.2.4 Measurement of thrombin generation by Calibrated Automated Thrombogram®

See corresponding section in Paper II.

All thrombin generation measurements were performed by the author.

The mixture of PPP reagent (trigger) and PPP used in the assay contained 5 pM tissue factor and 4 μM phospholipids.

2.2.5 Duration of treatment with anticoagulants

The duration of anticoagulation varied for the patients included in the study and was decided on an individual basis by the treating physician according to the guidelines for treatment of VTE in Sweden. Proximal VTE (provoked or unprovoked) are usually treated for six months and a provoked distal VTE for three months. Duration depended on the presence of DVT and/or PE, localization of thrombosis, previous VTE, hereditary and/or acquired thrombophilia etc. All patients were treated with warfarin. Warfarin treatment was initiated under overlapping treatment with low molecular weight heparin (LMWH) according to the guidelines mentioned above.

2.2.6 Statistical Analysis

Parametrical methods (two sample t-test) were used to perform the analyses when $n>30$. When $n<30$, non-parametrical methods (Mann-Whitney *U* test) were employed. P values <0.05 were considered statistically significant.

Pearson's correlation analysis was used for the thrombin generation markers at time of inclusion as well as after discontinuation of treatment with anticoagulants.

The procedure for follow-up and the methods for identifying FV Leiden mutation and for diagnosing DVT and PE are described in the corresponding section of Paper II.

2.3 Results

2.3.1 Thrombin generation in relation to recurrence, presence of provoking factor for the initial thromboembolic episode, gender and the presence of factor V Leiden

The patients with recurrences had longer lagtime ($p<0.006$, 5.2±4.1 min vs. 4.2±1.6 min) and ttpeak ($p=0.034$, 8.6±5 vs. 7.2±2.2) than the patients without recurrences. The patients who did not experience recurrences during follow-up had higher, but not statistically significant total (ETP, $p=0.111$, 1664±519 vs. 1490±535 nM*min) and maximum (peak, $p=0.058$, 302±92 vs. 260±124) thrombin generation compared with the patients with recurrences (Table 2).

The aforementioned pattern was repeated, with statistically significant results, in the subgroup with an initial unprovoked VTE. The patients with at least one recurrence during follow-up had longer lagtime ($p<0.001$, 5.6±4.6 vs. 4.2 ±1.5) and ttpeak ($p=0.029$, 9±5.7 vs. 7±2.4) than had the patients without recurrences and those without recurrences had significantly higher ETP ($p=0.038$, 1645±563 vs. 1384±551) and peak ($p=0.024$, 298±92 vs. 242±130) than those with recurrences (Table 2). No such differences were observed in the group of patients with an initial provoked VTE.

Neither gender not the presence of FV Leiden had any effect on the results. Age was not studied as a confounder, as the cohort consisted solely of adult patients.

2.3.2 Thrombin generation at the time of the inclusion and after discontinuation of treatment with anticoagulants

Of the 115 patients included in this cohort, thrombin generation data from both before and after treatment were available for 58 (Table 3).

The only statistically significant difference between the thrombin generation markers at inclusion and after discontinuation of anticoagulants was that lagtime was longer at inclusion ($p=0.047$). The correlation analysis showed a weak correlation for both ETP and peak at inclusion and after discontinuation of anticoagulants (Pearson correlation 0.273 and 0.273, respectively). Mann-Whitney´s test for the thrombin generation markers after discontinuation of treatment showed somewhat higher peak thrombin for the patients that experienced recurrences ($p=0.059$) compared with those that did not. These results contradict the results from the first analysis, i.e. that peak thrombin at the time of the inclusion was greater in patients without recurrences.

2.4 Discussion

Due to the variety of the number of patients in each group and of the statistical methods (parametric and non-parametric) that were used, the results in Tables (2) and (3) are expressed as median (range) whereas the results in Paper II are expressed as mean±SD.

Four of the patients included in this study were heterozygous for the PT-G20210A mutation (see Table 1). This factor was not taken into consideration into during the analysis and interpretation of the results because i) the number of patients with this mutation was very low and ii) it has been shown that thrombin generation does not differ among carriers of the mutation and controls, even in the presence of acute thrombosis (Paper I).

The unique design of this study makes comparisons and associations with other studies in the field difficult. Instead of blood sampling following discontinuation of anticoagulant treatment, thrombin generation was tested at the time of the acute thromboembolic episode. This allows for the results to be applied as early markers. At the same time, however, it raises questions about the effect of inflammation on thrombin generation markers.

The coagulation and inflammation system are interacting very closely [94] and an acute thromboembolic episode is an expression of acute inflammatory activation. This hypothesis is indirectly supported by the results obtained following discontinuation of anticoagulants, showing reversal of the thrombin generation profiles in relation to the outcome of the follow-up. Unfortunately, due to the small amount of available plasma it was not possible to analyze inflammation markers for those patients.

As in other studies [92, 80] on cohorts with high prevalence of the FV Leiden mutation, no thrombomodulin was added to the thrombin generation assay. This was decided so as not to decrease the sensitivity of the CAT® assay for recurrent venous thrombosis due to the effect of the common FV Leiden mutation on the assay result (18), as the presence of FV Leiden in its heterozygous variant has not been associated with significant increase in the recurrence risk by most studies in the field [95, 96].

The blood was centrifuged only once. To test whether residual platelet activity could have any significant effect on the results, 16 randomly picked samples (8 from patients with FV Leiden and 8 from patients without FV Leiden) were centrifuged once again following thawing (at 2500 g for 15 min) and thrombin generation in the double-centrifuged sample was measured. No statistically significant differences (Mann-Whitney *U* test) were found between the results from the samples centrifuged once and the samples centrifuged twice [lagtime (min): 3.6 (2.3–5.3) vs. 3.8 (2.5–7.7) p=0.742, ETP (nM*min): 1628 (1002–2220) vs. 1578 (1167–2149) p=0.547, peak (nM): 300 (85–460) vs. 280 (205–409) p=0.228, ttpeak (min): 6.3 (4.1–7.7) vs. 6.7 (5.5–11), p=0.869].

One of the strengths of the study is its long follow-up time (7 years), during which no patient was lost to follow-up. The main weakness is the inability to confirm the hypothesis about the effect of inflammatory markers on the "time" parameters of the thrombin generation assay (lagtime, ttpeak). As mentioned in 1.4 (Paper I, Discussion) it is rather paradoxical that prolonged initiation of the propagation phase, indirectly hinting at hypocoagulability, would be associated with recurrences during follow-up but even with a first-time thrombosis (Paper I), strongly suggesting the contribution of other factors. In a study by Smid et al [62] it was shown that patients with an acute myocardial infarction who experienced a recurrent episode during a 12-month follow-up had non-significant lower ETP and peak at the time of the acute episode compared to their counterparts without recurrence. F1+2 was however higher. The authors hypothesized that the effect of acute inflammation and vessel injury could have altered the results, as well as the effect of TFPI, which was shown to have a negative correlation with ETP. In the present study, TFPI was not measured, and the effect of vessel injury is not expected to be as predominant in patients with venous thrombosis as in patients with atherosclerosis. It does, however, provide an interesting aspect, possibly highlighting common pathways in the pathogenesis of arterial and venous thrombosis.

The fact that the differences in thrombin generation were most prominent in the patients with an initial unprovoked episode underlines the heterogeneity of this group. Whether undiscovered genetic factors or inadequately evaluated acquired risk factors contribute to that is open to speculation. However, the difficulties in accurately predicting the risk for recurrence and deciding on the length of treatment with anticoagulants for a patient without known risk factors stress the significance of finding new, reliable markers which was also the basis of this study. The fact that distinctly different thrombin generation profiles were associated with different clinical outcomes indicates a certain clinical relevance for those markers.

2.5 Conclusion

At the time of an acute thromboembolic episode prolonged clotting time and ttpeak seem to be associated with higher risk for recurrence during a 7-year follow up, whereas patients with higher total and maximum thrombin generation were at lower risk for recurrences. Those observations were particularly evident for the patients who had an initial unprovoked episode. The association of distinct thrombin generation profiles with different clinical outcomes during follow-up could pave the way for larger studies with similar design in order to evaluate thrombin generation markers as early markers for recurrence risk. At the same time, the effect of inflammation should be co-evaluated.

2.6 Tables

	n	age	M/F	previous VTE	FV Leiden	PTG20210A	DVT/PE (inclusion)
patients with recurrences (all)	40	62 (22–88)	20/20	7	16	2	34/6
patients without recurrences (all)	75	65 (20–86)	29/46	27	19	2	38/12
provoked VTE	39	54 (20–82)	11/28	8	12	3	36/3
unprovoked VTE	76	66 (22–88)	38/38	26	23	1	61/15
provoked VTE with recurrence	11	58 (24–82)	3/8	1	3	0	11/0
provoked VTE without recurrence	28	54 (20–82)	8/20	7	9	1	25/3
unprovoked VTE with recurrence	29	62 (22–88)	17/12	6	13	2	23/6
unprovoked VTE without recurrence	47	67 (24–86)	21/26	20	10	1	13/9

TABLE 1. *Characteristics of the patients included in the study.*

	lagtime (min)	ETP (nM*min)	peak (nM)	ttpeak (min)
patients with recurrences (all)	3.6 (2–11)	1729 (544–2262)	303 (59–490)	6.5 (5–21)
patients without recurrences (all)	3.8 (2–10)	1673 (903–2851)	308 (134–535)	6.7 (4–17)
provoked VTE	3.9 (2–10)	1778 (968–2851)	323 (117–477)	6.5 (4–12)
unprovoked VTE	3.7 (2–11)	1633 (544–2569)	302 (59–535)	6.9 (4.3–21)
provoked VTE with recurrence	3 (2.3–7)	1833 (1066–2262)	330 (117–439)	6 (5–12)
provoked VTE without recurrence	4.1 (2–10)	1732 (968–2851)	320 (134–477)	6.6 (4–12)
unprovoked VTE with recurrence	3.7 (2–11)	1683 (544–2143)	303 (59–490)	6.9 (4.8–21)
unprovoked VTE without recurrence	3.7 (2.2–9.3)	1614 (903–2569)	301 (135–535)	6.8 (4.3–11)

TABLE 2. *Thrombin generation markers for the patients included in the cohort. The results are reported as median (range).*

n=58	TG upon inclusion	TG after discontinuation of anticoagulants
lagtime (min)	3. 9 (2–7.6)	3.6 (2–10.5)
ETP (nM*min)	1496 (465–2221)	1504 (833–2363)
peak (nM)	277 (80–477)	263 (130–451)
ttpeak (min)	6.9 (4–17)	7.1 (4.2–14)

TABLE 3. *Thrombin generation (TG) markers upon incluson and following discontinuation of treatment with anticoagulants. The results are reported as median (range).*

3 Thrombin generation during the menstrual cycle. Paper III.

3.1 Introduction

3.1.1 Overview of the menstrual cycle

The menstrual cycle is defined as cyclic changes in the uterus and ovaries, which are essential for the ovulation and the preparation of the uterus for pregnancy [97]. The average length of the menstrual cycle is 28 days with an average range of 26–35 days. The beginning of each cycle is counted from the first day of menstrual bleeding. Each cycle consists of three phases, which can be classified according to the changes in either the endometrial lining of the uterus (uterine cycle) or the follicles of the ovary (ovarian cycle) [97]. The uterine cycle consists of the menstruation, proliferative and secretory phase, whereas the ovarian cycle consists of the follicular phase, the ovulation and the luteal phase [97].

In the uterine cycle, the menstruation corresponds to cycle day (cd) 1–5, the proliferative phase to cd 6–14 and the secretory phase to cd 15–28. In the ovarian cycle, the follicular phase corresponds to cd 1–13, ovulation to ~cd 14 and the luteal phase to cd 15–28 [97, 98].

As in Paper III, the nomenclature corresponding to the ovarian cycle is used in this chapter.

The cyclic changes of the sex hormones oestradiol and progesterone are essential for the menstrual cycle. Oestradiol rises during the follicular phase following the emergence of the ovulatory dominant follicle, reaching its peak on cd 13–15. After ovulation, oestradiol concentrations increase up to the mid-luteal phase and afterwards decline [99]. Progesterone levels reach peak levels about six days following ovulation (cd 21–25) and decreases to preovulatory levels at the beginning of the follicular phase (cd 1–8) [99].

3.1.2 The haemostatic system during the menstrual cycle

Variations in the levels of female sex hormones are known to affect the coagulation cascade [100]. Additionally, intact haemostatic potential is essential for the control of menstrual bleeding, including cessation of bleeding [101]. This knowledge has lead to a number of studies attempting to evaluate the physiological changes in the coagulation system during the menstrual cycle. Both the design and results of those studies are diverse [102].

Bleeding disorders have been reported as the underlying cause of menorrhagia in about 20% of the women investigated [102]. Platelet dysfunction has been reported as the most prevalent bleeding disorder among women with menorrhagia, with von Willebrand disease being the second most prevalent (13%) [103, 104].

Platelet function during the menstrual cycle has been scarcely studied. It appears that platelet function is increased during the luteal phase [105, 106]. The variations of von Willebrand factor, however, have been extensively investigated [102]. About one half of those studies have found cyclic variations in the levels of von Willebrand factor, with the lowest concentrations observed during the early follicular phase (cd 1–7) [102]. However, as not all studies reported consistent results, no recommendation could be issued for a specific time-point suitable for blood sampling for women under investigation for von Willebrand disease.

Two studies reported levels of FVIII as lowest during the early follicular phase. However, the majority of the studies on FVIII reported no differences [102]. Fibrinogen is another coagulation factor that has been extensively studied; in a recent review, Knol et al [102] report that 20 studies on variations in fibrinogen during the menstrual cycle have been published. Of those, 8 reported variations in fibrinogen (6 reported the lowest levels of fibrinogen during the luteal phase and 2 during the follicular), however the remaining studies reported no variations [102]. Activated FVII has been reported to be higher during the follicular phase according to one study [107].

Fibrinolysis markers have also been studied. In most of the papers published in the field plasminogen activator inhibitor 1 (PAI-1) has been measured, but the results were not consistent [108, 109] and most studies revealed no cyclic variation [102]. Two of five studies that measured D-dimer revealed cyclic variations, with the lowest D-dimer concentrations reported during the late follicular [110] or luteal phase [108]. The remaining studies, however, reported no significant fluctuations [102].

3.1.3 Thrombin generation during the menstrual cycle

Although thrombin is central to the coagulation cascade, data on thrombin generation during the menstrual cycle are scarce. Both TAT [111] and F 1+2 [105] are rather constant during the menstrual cycle. Thrombin generation measurements by CAT® have not previously been performed in this context.

3.1.4 Effect of physiological concentrations of hormones on the haemostatic system

Few studies have been performed on the effects of physiological concentrations of female sex hormones on haemostatic parameters, with research on the field focused mainly on the effects of OC on coagulation. However, as progestogen-only OC are considered safe for women with thrombophilia and/

or previous VTE [112], it is asserted that oestradiol has the greatest effect on haemostasis, contributing to hypercoagulability.

3.2 Paper III

3.2.1 Aim

The aim of this study was to measure thrombin generation in the follicular and luteal phase of a regular menstrual cycle and associate the results with the levels of selected haemostatic parameters, physiological concentrations of female sex hormones (oestradiol, progesterone), sex hormone binding globulin (SHBG), antimüllerian hormone (AMH) and the number of pregnancies and deliveries for the women in the cohort.

SHBG is a transport glycoprotein for oestradiol and progesterone and does not appear to fluctuate during the menstrual cycle [113]. The levels of SHBG increase dose-dependently upon oestrogen intake and decrease after administration of progestogen; accordingly, this carrier protein was proposed as a measure for the "total oestrogenicity" of combined hormonal contraceptives [114]. AMH has been used as a marker of ovarian reserve. Most of the studies on the cyclic fluctuations of AMH report no differences [115, 116].

3.2.2 Patients

The original study cohort consisted of 104 healthy women who volunteered for the study. Two of the participants withdrew their consent and were subsequently excluded from the study. Inclusion criterion was a history of regular menstrual cycles. The exclusion criteria were pregnancy, breast-feeding or hormonal therapy within two months prior to the study. The menstrual cycle length (in days) was 28.2±2 (mean + SD) with a range of 25–34 days. The number of bleeding days was 4.8±1.2.

None of the participants had suffered a thromboembolic event. However, eight had at least one first-degree relative with a previous thrombosis. The mean age of the participants was 31.7±8.6 and the mean BMI 23.1±3 (mean + SD). Eighty-six of the participants had at some point used combined oral contraceptives. Twelve subjects (11.7%) were heterozygous carriers of the FV Leiden mutation.

Fifty-three women (52%) were nulligravidae, 12 (11%) had one pregnancy, 16 (16%) two pregnancies, 7 (7%) three pregnancies, 6 (6%) four pregnancies, 5 (5%) five pregnancies, 2 (2%) six pregnancies and 1 (1%) eight pregnancies. Of the 49 (47%) women who had been pregnant at least once, 8 (16.5%) had delivered no children, 11 women (22.5%) had one child, 19 (39%) had two children, 7 (14%) had three children and 4 (8%) women had four children each. Nine women (9%) had at least one miscarriage.

3.2.3 Blood sampling and handling

The process for collecting and handling the blood samples is described in detail in the corresponding section of Paper III.

3.2.4 Measurement of thrombin generation by the Calibrated Automated Thrombogram®

See corresponding section in Paper III.

All thrombin generation measurements were performed by the author.

The final mixture of PPP reagent (trigger) and PPP used in the assay contained 5 pM tissue factor and 4 μM phospholipids.

Complete thrombin generation profiles (for both phases) were obtained for 73 women. Subjects were excluded form the thrombin generation analysis when measurements for both phases were not available. Twenty per cent of the missing profiles were the result of errors during the preparation of the sample or technical errors during thrombin generation analysis.

3.2.5 Measurement of hormones and SHBG

The methods employed for the measurement of oestradiol, progesterone and SHBG are described in the Methods section of Paper III.

The level of AMH in serum was determined by ELISA using a commercial kit (AMH Gen II ELISA; Beckman Coulter) with a detection limit of 0.6 pM, and within- and between-assay coefficients of variation of 4% and 5%, respectively. The normal standard range of serum AMH by this method at the Department of Clinical Chemistry, Karolinska University Hospital is 5–43 pM (0.7–6 mg/L).

3.2.6 Measurement of coagulation parameters

The methods employed for the measurement of fibrinogen, FII, FVII, FVIII, FX, von Willebrand factor, D-dimer and antithrombin are described in the Methods section of Paper III.

PAI-1 was measured by an ELISA method (Invitrogen™, Camarillo, CA, USA).

3.2.7 Statistical analysis

As the number of observations (n) was >30, a parametric test (paired t-test, $p<0.05$, CI95%) was used to determine the differences in the thrombin generation and coagulation markers during the two phases of the menstrual cycle. The associations between haemostatic markers, thrombin generation markers, hormones and SHBG were evaluated by simple correlation method for continuous data (Pearson's) and the least squares multiple regression analysis. The statistical methods are described in greater detail in the corresponding section of Paper III.

3.3 Results

3.3.1 Haemostatic parameters and thrombin generation

Total thrombin concentration, as expressed by ETP, was found to be higher during the luteal, compared to the follicular phase (1609.2±342.8 vs. 1524.5±283.2 nM*min, P=0.027) (Figure 1). FX was higher during the follicular phase (p=0.028, 1±0.1 kIU/L vs. 0.9±0.1 kIU/L). Both D-dimer and FVII were higher during the follicular phase (D-dimer p=0.067, 0.1±0.1 kIU/L vs. 0.07±0.06 kIU/L and FVII p=0.09, 0.9±0.2 kIU/L vs. 0.8±0.2 kIU/L). However the differences were not significant (Table 1).

3.3.2 Associations between thrombin generation markers and coagulation markers, hormones, SHBG, number of pregnancies and deliveries

Lagtime in the follicular phase was strongly associated, but not correlated with antithrombin (p=0.018, Figure 2) and marginally with von Willebrand factor (p=0.053) (Table 2). Ttpeak was associated, albeit not significantly, with PAI-1 (p=0.052, Figure 3) in the luteal phase (Table 3). No recurrent pattern of association was observed between the thrombin generation markers and the coagulation parameters, neither during the follicular (Table 2) nor the luteal phase (Table 3). No significant correlations were observed.

No significant or recurrent associations were found between thrombin generation, hormones, SHBG and indirect markers of fertility (number of pregnancies and deliveries) during the two phases of the menstrual cycle (Tables 4, 5).

Progesterone was shown to have significant associations with fibrinogen and FX (multiple regression analysis p=0.043 and p=0.033, respectively) and marginal association with FVIII (p=0.056) during the follicular phase (table 4). During the luteal phase progesterone was significantly associated with PAI-1 (Figure 4), FII and FVII (p=0.002 and p=0.034 respectively p=0.033, respectively) and marginally with D-dimer (p=0.07) (Table 5).

3.4 Discussion

It was shown that total thrombin concentration (ETP) in the luteal phase was higher than in the follicular phase. The hypothesis was that this could be explained by the effect of coagulation factors/markers on thrombin generation. It was expected that coagulation activity, as expressed by the levels of coagulation markers, would be enhanced in the luteal phase. However, this was not shown. On the contrary, FX and FVII were higher in the follicular phase, as was D-dimer. The lack of relation between the coagulation markers examined and thrombin generation markers was confirmed when no recurrent strong associations were found. The physiological levels of the hormones studied (oestradiol, progesterone and AMH) and of the transport glycoprotein SHBG did not have any significant effect on thrombin generation, either. However, it was shown that progesterone did have a stronger effect on coagulation during both

phases of the menstrual cycle than oestradiol had. This was an unexpected finding, as oestradiol has been asserted as the most influential hormone for haemostasis, as extrapolated by studies performed on the thrombogenicity of oestrogen-containing OC. On the contrary, case-control studies on the risk of thrombosis in women using progestogen-only pills or injectables suggest that there is little or no increase of the risk of thrombosis [117, 118, 119] although these studies were limited by the small number of participating women using these types of contraceptives. Furthermore, Kemmeren et al demonstrated favorable effects of progestogen-only OC on the risk markers of thrombosis, such as APCsr [120]. The effect of progesterone was more pronounced during the luteal phase, when thrombin generation was also higher. Progesterone, however, had no significant association with thrombin generation markers. As no significant correlations were found, a conclusion cannot be drawn as to the type of association involved. It is unclear whether thrombin generation is indirectly affected by progesterone, with the effect mediated by factors such as FII and FVII, which have been shown to be important for thrombin generation [25]. Larger studies are required in order to evaluate this unexpected finding and its potential significance.

Maintaining a normal haemostasis is essential for the prevention of excessive menstrual bleeding. During menstruation, the cessation of bleeding is achieved by platelet aggregation, fibrin deposition and thrombus formation [101]. TF and its binding to circulating FVIIa for the formation of the TF/FVIIa complex is central to the endometrial haemostasis, as in the systemic haemostasis. Endometrial haemostasis is different from haemostasis taking place for example on the skin following an injury, as there are fewer exclusively intravascular haemostatic plugs in the endometrium. A highly active fibrinolytic process participates in the regulation of normal menstruation. Large amounts of fibrin and fibrin degradation products are found in the menstrual fluid; in this study, higher D-dimer was found during the early follicular phase, which corresponds to the menstruation phase of the uterine cycle [97, 101], suggesting systemic rather than local hyperfibrinolytic activity.

As only 9 women had miscarriages, the number of pregnancies and deliveries as marker for fertility was not corrected for this factor.

Defining whether cycles were or were not ovulatory was a challenge.

In a previous study on the same cohort [121], a cycle was considered ovulatory if: i) the progesterone level in the luteal phase was above 15 nM/L and increased compared with the follicular phase level and ii) the oestradiol level was increased in the follicular compared with luteal phase.

Of the 102 women included in the study, 16 were assessed as having anovulatory cycles at the time of the sampling, as progesterone was <15 nM/L in the luteal phase and oestradiol was lower in the follicular than in the luteal phase. In addition, as 14 women had hormone levels that did not fulfill either criterion (i) or (ii), it could not be concluded whether they should be classified as

having anovulatory or ovulatory cycles. In [121] all 30 of those women were considered as having an anovulatory cycle and were subsequently excluded from the analysis. The aim of the study [121] was to evaluate APC resistance during the menstrual cycle. In the present study, the aim was to evaluate the haemostatic system, as represented by thrombin generation, coagulation and fibrinolysis markers during a regular menstrual cycle in relation to hormone levels and indirect markers of fertility. It has been reported that 40% of regularly menstruating women aged 20–40 years show luteal phase abnormalities; in one fourth of those women, low preovulatory oestradiol concentrations are also documented [122]. The presence of ovulation in the women included in the study was not assessed by methods such as transvaginal Doppler examination for measurement of the uterine blood flow, which is an indirect marker of endometrium receptivity [123] nor by measurement of the ovarian response to physiological gonadotropin stimulation, which is the assumption for normo-ovulatory cycles [115]. Surges of luteinizing hormone (LH) indicative of ovulation [98] were not measured either. Other ways of evaluating the presence of ovulation based on hormonal levels include defining a cycle as anovulatory if peak progesterone concentrations were <5 ng/mL on any given day during the cycle and no serum LH peak was observed during the mid- or late-luteal phase [124]. In [124] 42 of 509 regular cycles (8.3%) were reported to be anovulatory.

It is very difficult to accurately document ovulation. As none of the aforementioned methods for objective documentation of ovulation were applied in the present cohort, none of the 30 women with debatable ovulation were excluded.

About 12% of the women in the present cohort were heterozygous carriers of the FV Leiden mutation. This prevalence corresponds to the prevalence found in the Caucasian population [58], and it was not deemed necessary to modify the CAT® method by adding thrombomodulin.

In conclusion, the present study partly confirmed findings from other studies on the fluctuations of haemostatic markers during the menstrual cycle. The reason behind the different results of the studies in the field is open to speculation. It is known that haemostatic parameters are subject to intraindividual variations, which range from negligible to considerable depending on the marker studied. Therefore, it is expected that the results of studies on haemostasis during the menstrual cycle can be variable. The design of those studies varied, mostly in terms of the time of the blood sampling during the course of the two phases and the number of patients included. Additionally, different methods were used to measure the same parameter in different studies. However, relevant studies can offer valuable insights on the pathophysiology of menorrhagia as well as the effect of physiological concentrations of female hormones on the coagulation system. Such results could subsequently be extrapolated to reflect the effect of high doses of steroids, as contained in contraceptives and hormone replacement therapy.

3.5 Conclusion

This study provides evidence of different thrombin generation profiles in the two phases of the menstrual cycle, showing higher total thrombin production during the luteal phase. This finding cannot be explained by the effect of coagulation parameters that were studied and have been shown to fluctuate during the menstrual cycle. Indirect markers of fertility (AMH, number of pregnancies/deliveries) were not associated to the haemostatic potential either. Keeping in mind the close relationship between coagulation and inflammation and thrombin's role in inflammation, as well as the changes physiologically occurring in the uterine endothelium during the menstrual cycle, a logical next step would be to study the associations between inflammatory and vascular markers and coagulation during the follicular and luteal phase.

Additionally, progesterone was shown to be the most influential hormone, which was an unexpected finding. Further studies on larger cohorts are required in order to evaluate the significance of those findings, as well as the probability of extrapolating those results from the study of physiological concentrations of progesterone to the supraphysiological concentration of the progestogen included in oral contraceptives.

3.6 Tables

	follicular phase	luteal phase	p
lagtime (min)	2.59±0.4	2.67±0.5	0.261
ETP (nM*min)	1524.5±283.2	1609.2±342.8	0.027
peak (nM)	300.1±50	308.1±54.4	0.195
ttpeak (min)	5.2±0.7	5.3±0.0.7	0.715
PGN (nmol/L)	2.9*±4.3	31.9±22	
SHBG (nmol/L)	56.3	58.1	
oestradiol (pmol/L)	116.7±85	346.6±203.4	
AMH (ng/ml)	3.1±3.2	2.6±2.1	0.24
FII (kIU/L)	1±0.1	1±0.1	0.318
antithrombin (kIU/L)	1±0.07	1±0.08	0.337
fibrinogen (g/L)	2.6±0.4	2.9±1.7	0.215
D-dimer (mg/L)	0.1±0.1	0.07±0.06	0.067
FVII (kIU/L)	0.9±0.2	0.8±0.2	0.09
FVIII (kIU/L)	1±0.7	1±0.3	0.828
FX (kIU/L)	1±0.1	0.9±0.1	0.028
vWF* (kIU/L)	0.9±0.4	0.9±0.3	0.256
PAI-1 (μg/L)	2±1.8	2±1.8	0.14

TABLE 1. *Thrombin generation, hormone, SHBG and coagulation parameters for the patients included in the study. The values are expressed as mean±SD.*

[*] *VWF: von Willebrand factor.*

p	lagtime	ETP	peak	ttpeak
antithrombin	**0.018**	0.149	0.481	0.245
fibrinogen	0.919	0.493	0.457	0.570
D-dimer	0.493	0.600	0.456	0.580
PAI-1	0.367	0.189	0.511	0.676
FII	0.942	0.131	0.347	0.513
FVII	0.146	0.470	0.448	0.694
FVIII	0.786	0.730	0.788	0.649
FX	0.5	0.675	0.712	0.358
vWF	0.053	0.214	**0.024**	0.230

TABLE 2. *Results from regression analysis for the association between coagulation and thrombin generation markers during the follicular phase. The results are statistically significant when $p<0.05$ (marked in bold).*

p	lagtime	ETP	peak	ttpeak
antithrombin	0.149	0.389	0.245	0.603
fibrinogen	0.309	0.349	0.650	0.587
D-dimer	0.689	0.275	0.479	0.337
PAI-1	0.294	0.199	0.336	0.052
FII	0.323	0.199	0.333	0.284
FVII	0.892	0.892	0.838	0.741
FVIII	0.132	0.686	0.901	0.184
FX	0.230	0.205	0.650	0.175
vWF	0.699	0.952	0.459	0.648

TABLE 3. *Results from regression analysis for the association between coagulation and thrombin generation markers during the luteal phase. The results are statistically significant when p<0.05.*

p	oestradiol	progesterone	AMH	SHBG	gravida	para
lagtime	0.916	0.478	0.209	0.235	0.480	0.961
ETP	0.428	0.585	0.258	0.307	0.466	0.677
peak	1.000	0.730	0.306	0.597	0.303	0.670
ttpeak	0.849	0.875	0.475	0.669	0.538	0.848
antithrombin	0.225	0.277	0.579	0.626	0.294	0.086
fibrinogen	0.387	**0.043**	0.555	0.705	0.172	0.198
D-dimer	0.813	0.395	0.850	0.651	0.816	0.727
PAI-1	0.176	0.193	0.396	0.082	0.791	0.940
FII	0.439	0.210	0.493	0.159	0.830	0.199
FVII	0.116	0.245	0.690	0.223	0.388	0.597
FVIII	0.188	0.056	0.645	0.5	0.987	0.761
FX	0.178	**0.033**	0.446	0.06	0.730	0.657
vWF	0.881	0.812	0.176	0.892	0.594	0.915

TABLE 4. *Results from regression analysis for the association between coagulation and thrombin generation markers and hormones, SHBG, number of pregnancies (gravida) and births (para) during the follicular phase. The results are statistically significant when p<0.05 (marked in bold).*

p	oestradiol	progesterone	AMH	SHBG	gravida	para
lagtime	0.732	0.939	0.474	0.795	0.092	0.189
ETP	0.391	0.455	0.505	0.914	0.928	0.994
peak	0.232	0.259	0.296	0.686	0.841	0.990
ttpeak	0.697	0.204	0.460	0.636	0.200	0.260
antithrombin	0.338	0.585	0.055	0.478	0.184	0.153
fibrinogen	0.586	0.556	0.295	0.448	0.526	0.609
D-dimer	0.127	0.07	0.857	0.499	0.407	0.245
PAI-1	0.392	**0.002**	0.719	0.927	0.322	0.651
FII	0.382	**0.034**	0.550	0.732	0.418	0.520
FVII	0.574	**0.024**	0.092	0.667	0.945	0.703
FVIII	0.916	0.989	0.955	0.639	0.967	0.700
FX	0.546	0.858	0.374	0.643	0.803	0.951
vWF	0.425	0.317	0.739	0.620	0.732	0.624

TABLE 5. *Results from regression analysis for the association between coagulation and thrombin generation markers and hormones, SHBG, number of pregnancies (gravida) and births (para) during the luteal phase. The results are statistically significant when p<0.05 (marked in bold).*

3.7 Figures

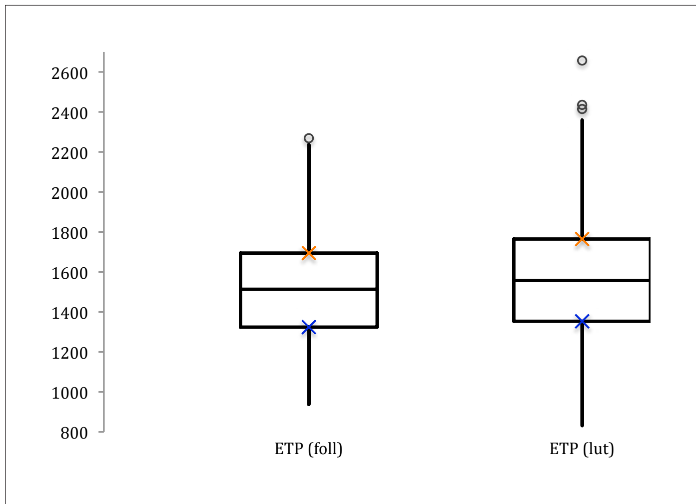

FIGURE 1. *ETP during the follicular and luteal phase of the menstrual cycle (1609.2±342.8 vs. 1524.5±283.2 nM*min, p=0.027).*

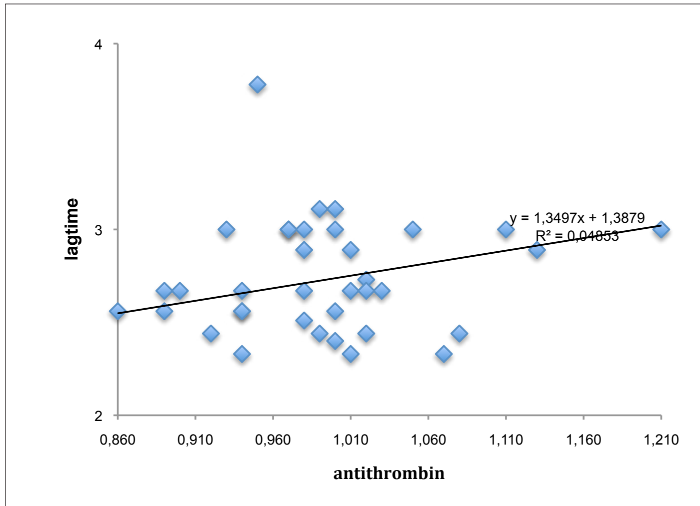

FIGURE 2. *Scatter plot matrix illustrating the correlation between lagtime and antithrombin during the follicular phase.*

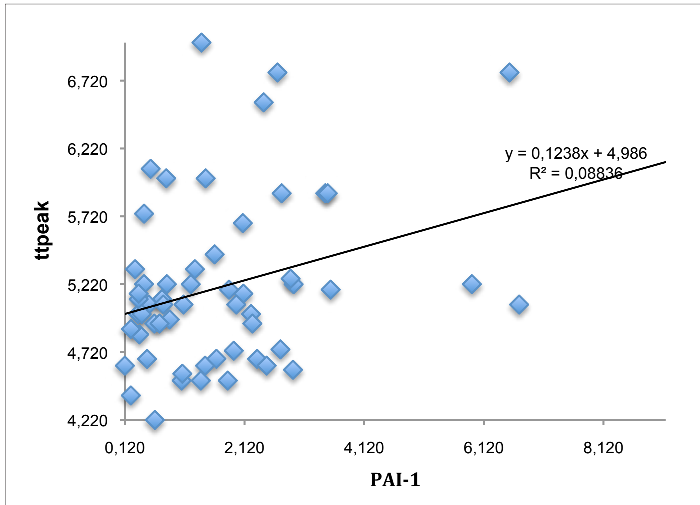

FIGURE 3. *Scatter plot matrix illustrating the correlation between ttpeak and PAI-1 during the luteal phase.*

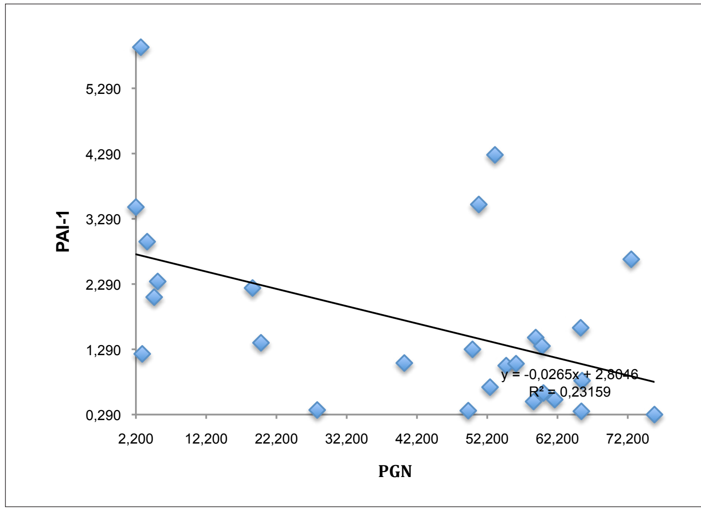

FIGURE 4. *Scatter plot matrix illustrating the correlation between PAI-1 and progesterone (PGN) during the luteal phase.*

4 Thrombin generation in patients with liver disease. Paper IV.

4.1 Introduction

4.1.1 Liver diseases

Portal vein thrombosis (PVT) is defined as an occlusion of the extra-hepatic portal vein, with or without concomitant obstruction of the intrahepatic vessels, superior mesenteric vein and splenic vein [125]. The actual prevalence of PVT is unclear. However, due to the increasing use of imaging techniques and routine surveillance, more cases of PVT have been reported in recent years. PVT can be associated with local risk factors [cirrhosis (cirrhotic-PVT, C-PVT), abdominal inflammation, infections, surgeries etc.] and systemic risk factors (thrombophilia, myeloproliferative syndromes etc.) [126]. C-PVT occurs mainly during the late stages of the disease [127], with a prevalence of ultrasound verified C-PVT of 25% [128].

Budd-Chiari syndrome (BCS) is a rare disease, characterized by obstruction of the hepatic venous outflow tract mainly due to thrombosis of the hepatic veins. Its aetiology has been associated with the presence of thrombophilia and myeloproliferative diseases. The rarity of the condition has not allowed for concise reports of its worldwide prevalence [129].

Cirrhosis is the morphological end result of many liver diseases and an important cause of morbidity and mortality [130]. It is caused by a variety of diseases, with hepatitis B being the most common, as well as non-alcoholic steatohepatitis, autoimmune hepatitis, metabolic liver diseases and primary sclerosering cholangitis [130]. The degree of cirrhosis is measured by the Child-Pugh score (CP). The CP score ranges between 5–15 and is calculated according to the presence and severity of ascites and hepatic encephalopathy, the prolongation of PT, and the levels of serum bilirubin and albumin. According to their CP scores, patients are classified into three classes (CP class A, B, and C with CP scores 5–6, 7–9, and 10–15, respectively) [131]. The degree of coagulopathy increases with increased CP score (CP class) [132].

4.1.2 Coagulopathy in liver disease

The liver is a pivotal organ for coagulation, as most of the coagulation factors (fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII and FXIII) and anticoagulant factors (antithrombin, PC, protein S) are synthesized in the liver [133]. The liver's central role in coagulation is underlined by the complexity of coagulopathy accompanying chronic liver disease. End-stage liver disease in particular is

characterized by decreased levels of most procoagulant factors, except for FVII and von Willebrand factor, as well as decreased levels of anticoagulant proteins [132]. Additionally, chronic liver disease is characterized by thrombocytopenia [134], which is, however, balanced by the high levels of von Willebrand factor that restores the effect of platelet adhesion to endothelium [135]. Hyperfibrinolysis has been reported, but its clinical significance, as manifested by bleeding complications, is uncertain [136].

The coagulopathy of chronic liver disease (CLD) is thus currently perceived as the product of a "rebalanced" haemostasis. CLD has been previously described as a prototype for bleeding disorders. This was based mainly on the fact that traditional coagulation tests such as PT-INR and aPTT are increased in such patients [132]. However, those tests reflect but a small part of haemostasis and are far from representative of the complex coagulation defects observed in CLD. Thrombin generation tests as global markers of the haemostatic potential have been employed in CLD and have shown that those patients are not ''naturally anticoagulated''; they are rather at increased risk for thrombotic complications [137]. In a large, nationwide, case-control Danish study it was shown that patients with liver disease had an increased RR of VTE [138].

4.1.3 Thrombin generation in liver disease

As mentioned earlier [8], binding of thrombomodulin to thrombin enhances the anticoagulant role of PC, effectively down-regulating thrombin. This is also an in vivo phenomenon, as adding thrombomodulin to plasma from healthy subjects decreases thrombin generation. This effect, however, is greatly inhibited in patients with CLD, leading to ''thrombomodulin resistance''. Thrombomodulin resistance is obvious when the thrombin generation markers are as expressed as ratios [markers measured in the presence]/[markers measured in the absence of thrombomodulin], with the resistance being higher the higher the ratio [132]. Thrombomodulin resistance is the result of markedly increased levels of FVII and decreased levels of PC, both observed in CLD [139].

In 2005 Tripodi et al [140] measured thrombin generation in the presence of thrombomodulin in 44 patients with cirrhosis (14 with CP class A, 16 with CP class B and 14 with CP class C) and compared the results against those obtained from 44 normal subjects. It was found that thrombin generation in the presence of thrombomodulin was nearly as high in cirrhotic patients as in controls, thus providing evidence of a rebalanced secondary haemostasis [140]. In patients with cirrhosis, FII is reduced [132]. FII is one of the procoagulant factors greatly affecting thrombin generation [25]. Tripodi et al [140] attributed their findings to a concomitant reduction in PC in cirrhotic patients, allowing for a rebalanced haemostasis in the presence of thrombomodulin, as it is under those conditions that PC exercises its full effect.

Gatt et al [141] designed a study to test the results from study [140] by measuring thrombin generation in 73 patients with cirrhosis and comparing the results to those obtained from 38 normal subjects. In [141], no thrombomodulin was used; the authors opted for using Protac®, a snake venom that activates PC in a manner similar to thrombomodulin. Total thrombin generation (ETP) and ttpeak were higher in cirrhosis patients when measured in the presence of Protac®, and Protac® resistance was evident in the group with cirrhosis (as expressed by the ratio marker measured in the presence/absence of Protac®) in a manner similar to thrombomodulin resistance in other studies [140]. Further studies have confirmed normal to increased haemostatic potential in patients with liver diseases [142].

Thrombin generation (in PRP) does not increase after addition of one adult standard platelet dose in thrombocytopenic patients [143] nor after mixing plasma from cirrhotic patients with plasma from normal subjects in a concentration corresponding to 10 ml/kg body weight [144]. Those results challenge the existing concept of routinely administering platelets or plasma to cirrhotic patients prior to invasive procedures. However, Tripodi et al [145] have previously shown that thrombin generation can be impaired in severe thrombocytopenia, justifying the role of platelet transfusion in patients with haemorrhages.

A study by Raffa et al [146] showed that thrombin generation measured in the presence of thrombomodulin was higher in patients with non-cirrhotic PVT (NC-PVT) than in normal subjects.

4.2 Paper IV

4.2.1 Aim

The aim of this study was to investigate the haemostatic potential in patients with different hepatic diseases and compare the results with those obtained from normal controls. Thrombin generation in the presence and absence of thrombomodulin as well as thrombomodulin resistance were evaluated.

Additionally, FVIII and antithrombin were measured as markers for procoagulant and anticoagulant activity, respectively, and high sensitivity C-reactive protein (hs-CRP) as a marker for inflammation in the patient cohort.

4.2.2 Patients

The original cohort for this study consisted of 59 patients with PVT diagnosed 1995–2009, 17 patients with BCS diagnosed 1988–2009 and 25 patients with cirrhosis diagnosed 2002–2010.

Nineteen patients in the cohort (PVT n=12, BCS n=2, cirrhosis n=1) were treated with LMWH. Those patients were excluded from the study, as there were no suitable controls treated with LMWH.

The final cohort consisted of 47 PVT patients (36 with NC-PVT and 11 with C-PVT), 15 patients with BCS and 24 patients with cirrhosis.

Of those, 15 patients with PVT (2 with C-PVT and 13 with NC-PVT) and 10 patients with BCS were under treatment with warfarin at the time of the blood sampling (Table 1).

The causes of the cirrhosis in patients with C-PVT were: hepatitis C virus (n=4), alcoholic liver disease (n=2), cryptogenic (n=2), autoimmune hepatitis (n=2), hepatitis B virus (n=1), and for the patients with cirrhosis and no PVT: alcoholic liver disease (n=10), non-alcoholic fatty liver disease (n=4), hepatitis C virus (n=4), autoimmune hepatitis (n=3), haemochromatosis (n=1), cryptogenic (n=1) and primary sclerosing cholangitis (n=1).

4.2.3 Controls for the thrombin generation measurement

Thrombin generation was measured in 21 healthy volunteers [8 male and 13 female, median age (range): 57 years (29–69)]. This group ("control group") was used as controls for the patients that were not treated with warfarin (Table 2).

Additionally, thrombin generation was measured in 25 patients with AF and who were treated with warfarin, denoted as "warfarin controls" (15 controls for the patients with PVT treated with warfarin and 10 controls for the patients with BCS treated with warfarin). Those controls did not suffer from liver diseases and each one was age-, gender- and PT-INR-matched to the corresponding patient (Table 2).

4.2.4 Blood sampling and handling

Blood samples were collected after an overnight fast from antecubital veins in sodium 1/10 volume of 0.13 mmol/L citrate, EDTA and serum tubes (Becton Dickinson, Meylan, France). The samples were centrifuged within two hours at 2500 g for 10 minutes and stored at -70°C pending analysis.

Following thawing, the citrate samples used for analysis of thrombin generation were centrifuged once more at 2500 g for 10 minutes.

4.2.5 Measurement of thrombin generation by the Calibrated Automated Thrombogram®

See corresponding section in Paper IV.

All thrombin generation measurements were performed by the author.

The mixture of PPP reagent (trigger) and PPP used in the assay contained 5 pM tissue factor and 4 μM phospholipids.

Soluble rabbit lung thrombomodulin (American Diagnostica Inc, Stamford, CT) was added at a final concentration of 4 nanomolar/L (nmol/L).

4.2.6 Statistical analysis

Continuous variables were expressed as medians and ranges and tested for statistical significance with the nonparametric Mann-Whitney *U* test. The least squares multiple regression analysis were used for evaluating the associations between markers. A two-sided p value of 0.05 or less was considered as statistically significant. Statistical analyses were performed with the SPSS software package, version 17.0 (SPSS, Inc, Chicago, IL).

The methods used to measure FVIII, antithrombin and hs-CRP are described in the corresponding section of Paper IV.

4.3 Results

The median (range) for thrombin generation markers for the patients and controls without warfarin treatment are presented in Table 2.

The results for thrombin generation refer to the measurements in the presence of thrombomodulin, unless otherwise specified.

The term "ratio" refers to the ratio of [thrombin generation marker measured in the presence]/[thrombin generation marker measured in the absence of thrombomodulin] unless otherwise specified.

4.3.1 Thrombin generation in the presence of thrombomodulin and thrombomodulin resistance for patients with PVT, C-PVT and NC-PVT without treatment with warfarin

Thrombin generation markers did not differ significantly between patients with PVT and NC-PVT and their respective controls. The ratios for ETP and peak were significantly higher for the patients with PVT compared with controls [ETP ratio: p=0.021, 0.5 (0.1–1) vs. 0.4 (0.2–0.8). Peak ratio: p=0.009, 0.6 (0.1–1.1) vs. 0.4 (0.2–0.8)]. No differences were observed for the ratios between the patients with NC-PVT and the controls.

However, ETP and peak were significantly higher in the group with C-PVT compared with controls (ETP: p=0.044, 882 (406–1274) nM*min vs. 593 (296–1406) nM*min, peak: p=0.020, 166 (102–206) nM vs. 117 (56–318) nM]. The ratios for ETP and peak were also higher in the C-PVT group compared with controls [ratio ETP: p=0.001, 0.9 (0.3–1) vs. 0.4 (0.2–0.8). Ratio peak: p=0.001, 0.9 (0.4–1) vs. 0.4 (0.2–0.8)].

ETP and peak in patients with C-PVT were higher than in patients with NC-PVT [ETP: p=0.068, non-significant, 882 (406–1274) nM*min vs. 432 (275–1689) nM*min. Peak: p=0.037, 166 (102–206) nM vs. 79 (35–257) nM]. The ratios for lagtime, ETP and peak were also higher in the C-PVT group compared with the group with NC-PVT [lagtime: p=0.007, 1 (0.9–1.1) vs. 0.9 (0.6–1.4)]. ETP: p=0.018, 0.9 (0.3–1) vs. 0.5 (0.2–0.9). Peak: p=0.033, 0.9 (0.4–1) vs. 0.5 (0.1–1.1)].

Neither the thrombin generation markers nor the ratios were significantly different for the different CP classes. ETP was associated with the CP class. However this did not reach statistical significance (p=0.106).

4.3.2 Thrombin generation in the presence of thrombomodulin and thrombomodulin resistance for patients with Budd-Chiari syndrome without warfarin treatment

None of the thrombin generation markers were significantly different between BCS patients and controls.

The ratios for lagtime, ETP and ttpeak were higher in the BCS group than were the ratios for the controls (Lagtime: p=0.012, 1 (0.9–1) vs. 1 (0.9–1.2). ETP: p=0.049, 0.5 (0.4–0.7) vs. 1 (0.9–1.2). Peak: p=0.077, 0.5 (0.4–0.8) vs. 0.4 (0.2–0.8)].

4.3.3 Thrombin generation in the presence of thrombomodulin and thrombomodulin resistance for patients with cirrhosis

ETP and peak were significantly higher in the group with cirrhosis, compared with controls [ETP: p=0.006, ETP 929 (237–1677) nM*min vs. 593 (296–1406) nM*min. Peak: p<0.001, 191 (39–250) nM vs. 117 (56–318) nM]. The same was observed for ETP and peak ratios [ETP ratio: p<0.001, 0.7 (0.2–1.3) vs. 0.4 (0.2–0.8). Peak ratio: p<0.001, 0.8 (0.2–1.3) vs. 0.4 (0.2–0.8)].

No differences were found when patients with C-PVT and cirrhosis patients (without PVT) were compared.

The ratios for ETP and peak showed weak associations with the CP class (multiple regression analysis, p=0.148 and p=0.137, respectively).

ETP was significantly associated with CP class (p=0.015), whereas peak exhibited a weaker association (p=0.094). ETP in the patients with CP class C was significantly higher than in class A patients [p=0.039, 1513 (1349–1677) nM*min vs. 903 (237–1518) nM*min], and slightly, but not significantly, higher than ETP in the patients with CP class B [p=0.079, 1187 (643–1409) nM*min].

4.3.4 Thrombin generation in the presence of thrombomodulin and thrombomodulin resistance for patients with PVT, C-PVT, NC-PVT, Budd-Chiari syndrome, cirrhosis and controls treated with warfarin

No differences were observed between the thrombin generation markers in the subgroup of patients with PVT, C-PVT, NC-PVT and BCS treated with warfarin and their respective controls with AF.

4.3.5 Thrombin generation in the presence of thrombomodulin and factor VIII in patients with PVT not treated with warfarin compared to patients with cirrhosis without PVT

Both ETP and peak were significantly higher in the group with cirrhosis than the group with PVT [ETP: p=0.007, 929 (237–1677) nM*min vs. 623 (275–1689) nM*min. Peak: p=0.007, 191 (39–250) nM vs. 103 (35–257) nM]. The ratios for lagtime, ETP and peak were similarly higher in the group with cirrhosis [lagtime ratio: p=0.017, 1 (0.6–1.4) vs. 1 (0.5–3.8). ETP ratio: p=0.031, 0.7 (0.2–1.3) vs. 0.5 (0.1–1). Peak: p=0.020, 0.8 (0.2–1.3) vs. 0.5 (0.1–1.1)].

FVIII was similarly higher in the cirrhosis group [p=0.006, 2 (1.1–3.9) kIU/L vs. 2 (0.7–3.9) kIU/L].

ETP, peak, ETP ratio, peak ratio and FVIII were higher in the cirrhosis group than the patients with NC-PVT [ETP: p=0.001, 929 (237–1677) nM*min vs. 432 (275–1689) nM*min. Peak: p=0.006, 191 (39–250) nM vs. 79 (35–257). ETP ratio: $p<0.001$, 0.7 (0.2–1.3) vs. 0.9 (0.3–1). Peak ratio: $p<0.001$, 0.8 (0.2–1.3) vs. 0.5 (0.1–1.1)]. FVIII: p=0.001, 2 (1.1–3.9) kIU/L vs. 2 (0.9–3.9) kIU/L]. When the results from the cirrhosis group were compared with the group with C-PVT, no differences were observed.

4.4 Discussion

Patients with cirrhosis were shown to have higher thrombin generation than controls, independently of the presence of thrombosis. This was evident, not only when comparing thrombin generation markers between patients with cirrhosis without PVT and patients with C-PVT, but also between patients with cirrhosis and NC-PVT. Additionally, resistance to thrombomodulin, common among patients with CLD [139], was more evident in cirrhotic patients. Unfortunately, due to the small number of patients with advanced stages of cirrhosis included in the study (CP class 3, n=2), subgroup analysis on the degree of thrombomodulin resistance depending on the CP class could not be performed.

Such differences were not evident when thrombin generation was measured in the absence of thrombomodulin, thus confirming the original observations by Tripodi et al [140] that thrombin generation tests in patients with liver diseases cannot be accurately interpreted when no thrombomodulin is added.

No differences were observed between thrombin generation markers in patients and controls treated with warfarin, indicating that the haemostatic potential of the patients with BCS and PVT was not additionally limited prior to initiation of the treatment. The hypercoagulability caused by the presence of CLD in C-PVT is balanced by the effect of warfarin. An indirect conclusion could be that patients with liver disease and treated with warfarin appear not to have higher bleeding risk than controls (without hepatic diseases) treated with warfarin when the PT-INR is in the recommended therapeutic interval.

However, thrombin generation is heavily affected by anticoagulants [27] and there are no available data on the PT-INR of the patients with BCS and PVT prior to initiation of the treatment. This renders the conclusions from those observations limited.

The finding of hypercoagulability associated with cirrhosis is indirectly confirmed by the results from the BCS group. Despite the presence of thromboembolism, no hypercoagulability was evident. However, thrombomodulin resistance that characterizes patients with CLD was present even in patients with BCS. The small number of patients with BCS in this study (n=15 and 10 were treated with warfarin) limits further conclusions concerning the optimal anticoagulant treatment of such patients. However, due to the rarity of BCS [147] even observations from small cohorts can be valuable.

Raffa et al [146] reported that patients with NC-PVT have higher ETP than controls, which conflicts with the results presented above. The authors measured ETP using the same method and experimental conditions, including the final concentration of thrombomodulin (4 nM). However, blood sampling in [146] took place at the time of diagnosis, whereas in the present study, the median time between diagnosis and blood sampling was 46 months. One hypothesis that can explain the diversity between the results would be that the acute inflammatory effect at the time of the thrombosis could have affected the results (see also Papers I, II).

As illustrated in Table 3, the range of the values of thrombin generation markers is wide, both for the patients with liver diseases and for the healthy controls. As mentioned previously in this thesis (Part 1, section 2.4), there is no established normal range for thrombin generation markers. Subsequently, each laboratory usually uses its own “reference range”. Establishing a reference range is even more difficult for patients with liver diseases, as the levels of coagulation factors affecting thrombin generation might differ significantly, depending on the type and severity of the disease, and demands a large number of patients.

Overall, the main differences, both in thrombin generation markers and ratios, were observed in the “concentration” parameters (ETP and peak), whereas the “time” parameters (lagtime, ttpeak) did not differ significantly, neither among the patient group nor in the controls. This observation is of particular interest. Both the lagtime (clotting time) and the ttpeak have been shown to express the bleeding risk in patients with rare bleeding disorders [148]. If those results were to be extrapolated to patients with coagulopathy of liver disease, which has long been considered mainly as a bleeding disorder [132], it could lead to revisiting the current clinical practice of routinely administering blood products to patients with CLD prior to invasive procedures in order to minimize the risk of bleeding. The value of routine transfusions has already been challenged [143, 144] and revisiting this clinical practice would spare unnecessary trans-

fusions, as well as avoiding adverse transfusion-related reactions, unmotivated delay in surgeries etc.

4.5 Conclusion

Hypercoagulability, as expressed by thrombin generation measured in the presence of thrombomodulin and thrombomodulin resistance are dependent on the presence of cirrhosis and not on the presence of thrombosis. Patients with C-PVT, therefore, are hypercoagulable and could benefit from longer treatment with anticoagulants. The lagtime did not differ between the patients with liver diseases and the controls. This could indicate that patients with liver diseases have a low risk of bleeding, and are not routinely in need of transfusions, for example prior to an invasive procedure.

4.6 Tables

	PVT	C-PVT	NC-PVT	BCS	cirrhosis	controls
n	47	11	36	15	24	21
male/female	27/20	8/3	19/17	7/8	15/9	8/13
age	56 (24–80)	60 (35–74)	56 (24–80)	31 (24–57)	57 (28–73)	57 (26–69)
warfarin treatment	15	2	13	10	0	N/A
PT-INR [1]	1 (0.9–2)	1 (1–1.4)	1 (0.9–2)	1 (1–1.2)	1.2 (1–1.9)	N/A
CP-class A/B/C (n)	N/A	6/3/2	N/A	N/A	16/6/2	N/A

TABLE 1. *General characteristics of the patients and controls included on the study. The values are expressed as median (range).*

[1] *For patients without treatment with warfarin.*

	patients with PVT	patients with BCS	controls for PVT	controls for BCS
n	15	10	15	10
male/female	7/8	5/5	7/8	5/5
age	56 (36–70)	32 (24–56)	57 (50–64)	58 (43–63)
PT-INR	2.4 (1.5–3.4)	2.6 (1.6–3.5)	2.4 (1.5–2.8)	2.5 (2.4–2.7)

TABLE 2. *General characteristics of the patients and controls treated with warfarin. The values are expressed as median (range).*

	PVT	C-PVT	NC-PVT	BCS	cirrhosis	controls
n	32	9	23	5	24	21
lagtime+TM (min)	3.2 (1.5–28)	2.8 (1.5–4.3)	3.3 (1.8–28)	3.6 (3–4)	3.3 (2.7–15.2)	3.5 (2.3–5.3)
ETP+TM (nM*min)	636 (275–1689)	882 (406–1274)	432 (275–1689)	620 (470–1092)	929 (237–1677)	593 (296–1406)
peak+TM (nM)	103 (35–257)	166 (102–206)	79 (35–257)	102 (84–193)	191 (39–250)	117 (56–318)
ttpeak+TM (min)	6.1 (3.8–33)	5.7 (3.8–7.2)	6.4 (4.2–33)	6.7 (5.7–7.2)	6 (4.5–18.5)	6 (4.7–8.5)
lagtime-TM (min)	3.5 (1.5–25)	2.8 (1.5–3.8)	3.5 (1.8–25)	3.6 (3–4.5)	3.2 (2.5–7.5)	3.3 (2.3–5.3)
ETP-TM (nM*min)	1341 (388–2609)	991 (894–1730)	1425 (388–2609)	1263 (920–2060)	1352 (857–2006)	1585 (1108–2244)
peak-TM (nM)	198 (33–345)	196 (176–244)	79 (33–345)	210 (172–314)	220 (92–307)	280 (203–388)
ttpeak-TM (min)	6.9 (3.5–31)	6.1 (3.5–7.8)	7.1 (4.2–31)	9.7 (7.8–31)	6 (4.8–10)	6.3 (4.7–8.5)
lagtime ratio	1 (0.6–1.4)	1 (0.9–1.1)	0.9 (0.6–1.4)	1 (0.9–1)	1 (0.5–3.8)	1 (0.9–1.2)
ETP ratio	0.5 (0.1–1)	0.9 (0.3–1)	0.5 (0.2–0.9)	0.5 (0.4–0.7)	0.7 (0.2–1.3)	0.4 (0.2–0.8)
peak ratio	0.6 (0.1–1.1)	0.9 (0.4–1)	0.5 (0.1–1.1)	0.5 (0.4–0.8)	0.8 (0.2–1.3)	0.4 (0.2–0.8)
ttpeak ratio	0.8 (0.4–2.9)	0.8 (0.4–1.1)	0.8 (0.6–0.8)	0.6 (0.2–0.9)	1 (0.6–1.5)	0.4 (0.2–0.8)

TABLE 3. *Thrombin generation markers and ratios [marker measured in the presence]/[marker measured in the absence of thrombomodulin] for patients and controls without warfarin treatment. The values are expressed as median (range).*

5 Thrombin generation in patients with mild factor VII deficiency. Paper V.

5.1 Introduction

5.1.1 Factor VII deficiency

FVII is a vitamin K-dependent serine protease synthesized in the liver [149]. The human FVII protein is encoded by the *F7* gene (Gene ID: 2155) located on the long arm of chromosome 13, specifically 13q34, and contains nine exons [150]. The interaction between FVIIa and TF initiate thrombin generation via the extrinsic pathway [151].

Deficiency of FVII is a congenital bleeding disorder inherited in the autosomal recessive manner and is the most common among the rare bleeding disorders with a prevalence of about 1:300,000–1:500,000 [152, 153]. The laboratory finding of an isolated, spontaneously prolonged PT-INR either by chance or during investigation of a patient with bleeding symptoms triggers the suspicion of a vitamin K-dependent factor deficiency if the liver function is normal [132] and there is no deficiency of vitamin K. The prolongation of PT-INR in patients with FVII deficiency is variable and usually dependent on the level of FVII [154]. FVII deficiency is not believed to be associated with complete absence of functional FVII, as the results from studies in knockout mice suggest that a complete lack of FVII is incompatible with life [155].

The bleeding symptoms in patients with FVII deficiency are characterized by clinical heterogeneity and are not predicted by the levels of FVII [154]. Thirty per cent of the patients with FVII deficiency are asymptomatic [154]. The remainder have symptoms that vary from mild, mimicking the symptoms of patients with platelet function disorders (epistaxis, easy bruising, gum bleeding) to more severe symptoms (haemarthrosis, muscle haematomas, central nervous system bleeding, gastrointestinal bleeding) [156]. Menorrhagia is a common symptom amongst females [154].

5.1.2 Measurement of FVII by using different thromboplastins

In 1978, Girolami et al [157] reported that FVII levels in patients with the FVII Padua mutation were different depending on the origin (species) of thromboplastin used. FVII Padua is caused by an arginine (Arg) 304 to glutamine (Gln) substitution. In this variant, FVII activity was 6–8% of normal using rabbit brain thromboplastins (r-thromboplastin) and normal using ox brain throm-

boplastins. Intermediate values around 30–40% of normal were obtained using human placenta or human recombinant thromboplastin (h-thromboplastin) was used [158]. Those findings have been confirmed by other studies [159, 160, 161].

Other mutations associated with FVII deficiency have been described. FVII Nagoya is the result of a novel amino acid substitution of Arg304 to tryptophan (Trp). Patients with FVII Nagoya exhibit a lower FVII procoagulant activity when tested in a one-stage assay using rabbit, simian, or human thromboplastin, but display nearly normal activity when bovine thromboplastin is used [162]. In patients with FVII Shinjo, which is the result of the Arg79 to Gln substitution, normal FVII activity is obtained using human placenta thromboplastin but low activity when rabbit or bovine brain thromboplastin is used [163].

5.1.3 Thrombin generation in patients with FVII deficiency

Data on thrombin generation in patients with FVII deficiency are scarce. Tran et al [164] studied thrombin generation in 12 patients with FVII deficiency; 10 had an increased haemorrhagic diathesis and the remaining 2 were asymptomatic. Thrombin generation did not differ among those patients.

According to Al Dieri et al [165], 2% FVII is enough to generate 50% of normal ETP, and the bleeding tendency in patients with rare bleeding disorders is absent or mild when ETP is higher than 30% of normal.

Recently, Zekavat et al [148] showed that the “time“ parameters of thrombin generation assays, namely lagtime, ttpeak and start tail, can be used as markers for bleeding risk in patients with rare bleeding disorders.

5.2 Paper V

5.2.1 Aim of the study

This study was initiated after the accidental finding that some patients investigated for suspected FVII deficiency in the Coagulation Laboratory of the Clinical Chemistry Department of the Linköping University Hospital, Sweden had pronounced to slightly discrepant levels of FVII depending on the species of thromboplastin used in the assay. DNA analysis was performed to determine whether a specific mutation was responsible for this phenomenon. Thrombin generation in the presence of different thromboplastins was measured as a marker of haemostatic potential.

5.2.2 Design of the study

Between 2007 and 2011, 41 patients who were referred to the Coagulation Laboratory of the University Hospital in Linköping for investigation because of bleeding symptoms and/or spontaneously prolonged PT-INR were diagnosed as having FVII deficiency. FVII deficiency was defined as FVII activity level <0.6

kIU/L (reference range 0.6–1.6 kIU/L). The measurement of FVII activity was performed using r-thromboplastin, as this is the reagent routinely used in the laboratory.

FVII activity was measured in the presence of h-thromboplastin in 24 of the 41 patients as a quality control. The median ratio FVII measured in the presence of human/rabbit thromboplastin for those patients was 1.25. It was decided that a full genetic analysis of the *F7* gene and thrombin generation measurements [in the presence of h-thromboplastin (standard PPP reagent) and different dilutions of r-thromboplastin] would be performed for half of those cases where the ratios were highest.

As in Paper V, the use of the term 'thromboplastin' in the text corresponds to commercially manufactured tissue factor with phospholipids.

5.2.3 Patients

Of the 12 patients who were asked to participate in the study, 10 agreed. The main characteristics of the patients included in the study are shown in Table 1.

Exclusion criteria were age <18 years, inability to give informed consent and FVII deficiency of other definitively verified cause.

The study protocol was approved by the local Medical Ethics Committee. All patients signed a consent form prior to inclusion in the study.

The median ratio for FVII activity measured in the presence of human/rabbit thromboplastin for the 10 patients included in the study was 1.4. The patients participating in the study were unrelated, with the exception of #1 and #2 (mother and daughter).

Data on the bleeding phenotype were obtained by retrospective medical record review. Information was obtained on the following bleeding symptoms: epistaxis, easy bruising, oral mucosal bleeding, muscle haematoma, haemarthrosis, gastrointestinal bleeding, prolonged bleeding following minor trauma, central nervous system haemorrhage, peri/postoperative bleeding, bleeding during/following a tooth extraction, menorrhagia, postpartum haemorrhage and family history. The bleeding score was calculated according to the bleeding score used in Sweden, which is based on [166] and [167] and is described in detail in the corresponding section of Paper V. A bleeding score of 4 and above was considered pathological. The bleeding scores of the patients included in the study are shown in Table 1 (no data available for case #3) and their bleeding symptoms are described in detail in the corresponding section of Paper V.

5.2.4 Blood sampling and handling

Venous blood samples for analysis of DNA were collected in tubes containing EDTA (Vacutainer, Becton Dickinson, Meylen, France). Blood samples for measuring thrombin generation and FVII activity were collected in tubes contain-

ing 0.13 mM citrate (Vacutainer, Becton Dickinson, Meylen, France) and immediately centrifuged at 2000 g for 15 min. After removal of the supernatant, plasma was re-centrifuged for another 10 min at 2000 g. Platelet-poor plasma (PPP) and EDTA blood were stored at -70°C until analyzed.

5.2.5 Measurement of PT-INR

For details, see the corresponding section of Paper V.

One case (#6) was under treatment with coumarins (warfarin) at the time of inclusion in the study. The PT-INR and FVII presented in Table 1 are the results of measurements prior to initiation of treatment. There is no value for PT-INR measured in the presence of h-thromboplastin.

The methods for measuring FVII activity and genotyping of the F7 gene are described in the corresponding sections of Paper V.

5.2.6 Thrombin generation measurement by the Calibrated Automated Thrombogram®

See corresponding section of Paper V.

All thrombin generation measurements were performed by the author.

The final mixture of PPP reagent (trigger) and PPP used in the assay contained 5 pM tissue factor and 4 μM phospholipids.

To determine the concentration of rabbit thromboplastin corresponding to the concentration of the human TF (referred in this text as thromboplastin) used in the PPP reagent from Thrombinoscope®, a series of thrombin generation measurements in 12 healthy adult volunteers using various dilutions of r-thromboplastin were performed. The r-thromboplastin was diluted with Hepes buffer (prepared as described in the corresponding section of Paper V) and the dilutions used were: 1:50, 1:100, 1:200, 1:500 and 1:1,000. The results were compared with the results obtained from the thrombin generation measurement as described above, i.e. with a final h-thromboplastin concentration of 5 pM. It was shown (Mann-Whitney *U* test) that the results for all thrombin generation markers (lagtime, ETP, peak, ttpeak) did not differ significantly when measured with h-thromboplastin and r-thromboplastin diluted 1:200 [lagtime 3.4 min (3.1–5.2) vs. 3.6 min (3–4.2), p=0.120. ETP 1971 nM*min (1218–2994) vs. 2034 nM*min (1359–2681), p=0.729. Peak 308 nM (153–369) vs. 328 nM (229–399), p=0.356. Ttpeak 6.7 min (6.2–11) vs. 6.9 min (6.3–8.1), p=0.664]. Thrombin generation in the patients with FVII deficiency was measured with all the aforementioned dilutions of r-thromboplastin in order to study whether the low levels of FVII activity and the presence of specific mutations would affect the thrombin generation profiles usually observed with increasing thromboplastin concentrations.

5.2.7 Statistical analysis

Continuous variables were expressed as medians and ranges and were tested for statistical significance with the nonparametric Mann-Whitney *U* test. A two-sided p value of 0.05 or less was considered as statistically significant. Statistical analyses were performed with the SPSS software package, version 17.0 (SPSS, Inc, Chicago, IL).

5.3 Results

5.3.1 Mutations found in the patients

Six mutations, previously described as pathological, were found in the patients.

All but one patient (case #1) were carriers of the Arg353Gln mutation (homozygous: #6, #7, #9, #10 and heterozygous: #2, #3, #4, #5, #8).

Cases #1 and #2 (mother and daughter) and case #4 were heterozygous for the Arg79Gln mutation (FVII Shinjo).

Case #5 was heterozygous for the Arg304Gln mutation (FVII Padua).

Case #8 was heterozygous for the Gly375Glu mutation.

Cases #9 and #10 were heterozygous for the polymorphism Ala294Val.

Thus, 9 out of 10 patients had a mutation in common (Arg353Gln), 3 (#2, #4, #5) were compound heterozygous and 2 (#9 and #10) were heterozygous for one mutation (Ala294Val) and homozygous for another (Arg353Gln).

5.3.2 Thrombin generation in patients with FVII deficiency measured in the presence of human recombinant thromboplastin and different concentrations of rabbit brain thromboplastin

Lagtime was significantly higher when measured in the presence of 1:1,000 r-thromboplastin compared with h-thromboplastin ($p<0.001$, 4.5 min and 3.3 min, respectively), 1:50 r-thromboplastin ($p<0.001$, 4.5 min and 3 min, respectively), 1:100 r-thromboplastin ($p<0.001$, 4.5 min and 3.1 min, respectively), 1:200 r-thromboplastin ($p<0.001$, 4.5 min and 2.8 min, respectively) and 1:500 r-thromboplastin ($p<0.001$, 4.5 min and 3.3 min, respectively) (Table 2, Figure 1).

ETP was significantly higher when measured in the presence of 1:100 r-thromboplastin compared with h-thromboplastin ($p=0.004$, 2297 nM*min and 1700 nM*min, respectively), 1:50 r-thromboplastin ($p=0.003$, 2297 nM*min and 1540 nM*min, respectively), 1:200 r-thromboplastin ($p=0.009$, 2297 nM*min and 1771 nM*min, respectively), 1:500 r-thromboplastin ($p=0.031$, 2297 nM*min and 1783 nM*min, respectively) and 1:1000 r-thromboplastin ($p=0.005$, 2297 nM*min and 1690 nM*min. respectively) (Table 2, Figure 2).

Peak was significantly higher when measured in the presence of h-thromboplastin compared with 1:50 r-thromboplastin (p=0.001, 386 nM and 204 nM, respectively), 1:100 r-thromboplastin (p=0.024, 386 nM and 248 nM, respectively), 1:200 r-thromboplastin (p=0.002, 386 nM and 199 nM, respectively), 1:500 r-thromboplastin (p=0.003, 386 nM and 199 nM, respectively) and 1:1000 r-thromboplastin (p=0.001, 386 nM and 181 nM, respectively) (Table 2, Figure 3).

Ttpeak was significantly lower in the h-thromboplastin measurement compared with 1:50 r-thromboplastin (p=0.006, 5.3 min and 6.6 min, respectively), 1:100 r-thromboplastin (p=0.008, 5.3 min and 6.9 min, respectively), 1:200 r-thromboplastin (non significant, p=0.063, 5.3 min and 6.2 min, respectively), 1:500 r-thromboplastin (p=0.010, 5.3 min and 6.7 min, respectively) and 1:1000 r-thromboplastin (p<0.001, 5.3 min and 8 min, respectively). Ttpeak was significantly higher in the 1:1,000 r-thromboplastin measurement compared with the results obtained in the presence of the other r-thromboplastin dilutions (1:50 r-thromboplastin, p=0.003, 1:100 r-thromboplastin p=0.006, 1:200 r-thromboplastin p=0.001 and 1:500 r-thromboplastin p=0.004) (Table 2, Figure 4).

5.3.3 Thrombin generation in patients with FVII deficiency and controls measured in the presence of human recombinant thromboplastin and different concentrations of rabbit brain thromboplastin

When thrombin generation was measured in the presence of h-thromboplastin (standard thrombogram) ETP and ttpeak were significantly higher in the control group compared to the FVII deficiency group (ETP: p=0.028 1971 nM*min and 1700 nM*min, respectively. Ttpeak: p<0.001, 6.7 min and 5.3 min, respectively), whereas peak was higher in the FVII deficiency group (p=0.028, 386 nM and 308 nM, respectively).

When thrombin generation was measured in the presence of 1:200 r-thromboplastin, lagtime, peak and ttpeak were higher in the control group compared to the FVII deficiency group (lagtime: p=0.001, 3.7 min and 2.8 min, respectively. Peak: p=0.004, 328 nM and 199 nM, respectively. Ttpeak: p=0.003, 6.9 min and 6.2 min, respectively).

5.4 Discussion

Of the 10 patients included in the study, only 1 had a clearly pathological bleeding phenotype (case #1), whereas case #2 (daughter of case #1) had a bleeding score of 4, which is borderline normal. Both patients had mildly decreased FVII levels when measured by the method standardly employed by the laboratory (case #1: FVII 0.54 kIU/L, case #2: FVII 0.43 kIU/L). On the other hand, the patients with the lowest FVII levels (measured in the presence of r-thromboplastin, case #5: FVII 0.19 kIU/L, case #9 FVII 0.28, case #10 FVII 0.26) had no bleeding symptoms, despite having been exposed to "risk situ-

ations", such as caesarean section (case #5), multiple teeth extractions (case #5, case #10) and multiple trauma and surgery (case #9). This illustrates the diversity of the bleeding phenotype in patients with FVII deficiency, as well as the lack of correlation between the FVII levels and the severity of the symptoms.

In some cases, FVII deficiency has been paradoxically linked to thrombosis [168]. For example, DVT, splanchnic thrombosis and arterial thrombosis have been reported even in patients with severe FVII deficiency [169, 170, 171]. Mutations that lead to mild bleeding symptoms, such as FVII Padua (Arg-304Gln) and Arg294Val have been linked to thrombosis [172]. The mechanism behind thrombosis in those cases is unclear [168], but it has been suggested that most events are associated with an independent prothrombotic factor, such as surgery and the use of factor replacement therapy [173]. However, since, as mentioned above, some mutations lead to variable levels of FVII depending on the origin of the thromboplastin used, it could be assumed that the levels of FVII are underestimated and those patients subsequently overtreated with factor concentrates. Elevated FVII levels have been shown by some studies to be linked to cardiovascular disease [174, 175]. Another hypothesis is that polymorphisms such as Arg294Val and FVII Padua favor a second type of FVIIa, which binds TF more than the usual form [170]. Whether the Arg353Gln polymorphism, prominent in the present cohort, is linked to increased risk for a cardiovascular episode is controversial; some studies [176, 177] showed no significant association with cardiovascular disease, whereas others [178, 179] showed a slightly decreased risk for myocardial infarction. With the exception of case #3, where no clinical data were available, and case #6, no other patient had suffered a thrombosis, neither arterial nor venous. Case #6 had suffered venous thrombosis; however she had been diagnosed with polycythemia vera prior to the thrombosis and myeloproliferative syndromes are known risk factors for thromboembolism [180]. Thus, it is impossible to determine whether the presence of the Arg353Gln polymorphism contributed in any way to the development of thrombosis in this patient. However, as only one patient in this cohort is known to have had a thromboembolic episode, and this was additionally linked to an independent risk factor, the conclusion from this study is that the Arg353Gln polymorphism does not appear to increase the risk for either arterial or venous thrombosis.

The Arg353Gln polymorphism, which was found in 90% of the patients in this cohort, is rather common; Hunault et al reported a prevalence of about 13.1% in a population of Polish blood donors (n=99) [181]. Its prevalence in the Swedish population is unknown.

The ratio of FVII measured in the presence of human/rabbit thromboplastin was highest in cases #1, #2 and #5. In cases #1 and #2 the patients were heterozygous for FVII Shinjo and the patient #5 was heterozygous for FVII Padua. Both FVII Shinjo and FVII Padua have been associated with different results for FVII dependent on the type of thromboplastin used [162, 163] and the amino

acids Arg304 and Arg79 are located in the binding site for TF. However, none of the other polymorphisms detected in this study (Glu265, Ala294, Gly375 and Arg353) interact with TF [182]. Neither the Gly375Glu nor the Ala294Val variant have been associated with discrepancies in FVII levels depended on the type of thromboplastin used. The presence of compound heterozygotes limits the conclusions that can be drawn on a single mutation, however the presence of Arg353Gln alone [in both heterozygous (case #3) and homozygous (cases #6, #7) form] does not lead to pronounced differences in FVII activity. Therefore, it is concluded that the Arg353Gln polymorphism does not lead to discrepant results in FVII activity depending on the thromboplastin used in the reagent. Further studies are needed to clarify if the coagulation cascade, and in particular the activation of FX is influenced by any of those mutations in FVII.

Duchemin et al [32] have shown that, when measuring thrombin generation by CAT®, increases in TF concentration are associated with increases in ETP and peak thrombin, and decreases in lagtime. This pattern was partially confirmed by the presented results. Lagtime was highest when measured in the presence of the lowest thromboplastin concentration. Ttpeak was also higher with lower concentration of thromboplastin. ETP was highest in the presence of 1:100 r-thromboplastin, which corresponds to slightly higher thromboplastin than the concentration of 5 pM used in CAT®. Peak was highest when 5 pM r-thromboplastin was used. Therefore, the expected pattern of thrombin generation measured in the presence of different concentrations of thromboplastin was not affected by the origin of the thromboplastin used. The results indicate that, even in patients with FVII deficiency, lower concentrations of thromboplastin lead to a delayed and lower thrombin generation. Therefore, lower FVII activity does not lead to different reactivity for thromboplastin in vitro when thrombin generation is measured. It has to be kept in mind, however, than none of the patients included had a severe form of FVII deficiency. It is unclear whether these results for patients with mild FVII deficiency can be extrapolated to patients with a more severe form.

When thrombin generation was previously measured in FVII deficient plasma, ETP was up to 60–70% of normal with only 1% FVII [32]. ETP in the patient group was 86% of ETP in the control group, i.e. sufficient to sustain a normal haemostasis, which can explain the lack of bleeding symptoms in the patients.

Peak was higher in the patient group when thrombin generation was measured in the presence of h-thromboplastin, whereas it was lower in the patient group when measured in the presence of 1:200 r-thromboplastin. Rabbit and human TF are very similar [183], and the Arg 353 amino acid is not located in the area that interacts with TF, which eliminates this mechanism as a possible explanation. According to Brummel-Ziedins et al [25], peak thrombin in the presence of 5 pM h-thromboplastin is heavily influenced by FVII in the healthy population. It is unclear whether this pattern is altered in patients with FVII deficiency, or whether, in the presence of r-thromboplastin, thrombin generation is affected even by factors of the intrinsic pathway, such as FXI, or whether

the effect of other factors of the extrinsic pathway is greater, leading to higher peak thrombin concentration.

5.5 Conclusion

Although 90% of the patients included in this study had a mutation in common, it does not appear that this mutation causes diversities of the FVII levels when measurements are performed in the presence of thromboplastins of different origins. Total thrombin generation in patients with mild FVII deficiency was about 86% of the total thrombin generation in the control group, corresponding to the lack of severe bleeding symptoms in most of the patients. Thrombin generation patterns, as expected when increasing concentrations of thromboplastin are used, did not differ depending on the origin of thromboplastin in the patient group.

5.6 Tables

case	sex [a] age [b]	bleeding score [c]	PT-INR [d]	PT-INR [e]	FVII (kIU/L) [f]	FVII (kIU/L) [g]	ratio FVII (h/r –TP) [h]	nucleotide change	amino acid change
1	F/45	8	1.2	1.2	0.87	0.54	1.6	het.c. 416G>A	Arg79Gln (FVII Shinjo)
2	F/20	4	1.1	1.2	1.03	0.43	2.4	het.c. 416G>A het.c. 1238G>A	Arg79Gln (FVII Shinjo) Arg353Gln
3	M/46	–	1.2	1.2	0.74	0.54	1.4	het.c. 1238G>A	Arg353Gln
4	F/59	0	1.2	1.2	0.65	0.5	1.3	het.c. 973G>A het.c. 1238G>A	Glu265Lys Arg353Gln
5	F/40	-2	1.3	1.6	0.55	0.19	2.9	het.c. 1091G>A het.c. 1238G>A	Arg304Gln (FVII Padua) Arg353Gln
6	F/86	-1	n/a	1.2*	0.56*	0.43*	1.3	homo.c. 1238G>A	Arg353Gln
7	F/32	1	1.4	1.5	0.37	0.29	1.3	homo.c. 1238G>A	Arg353Gln
8	F/75	1	1.4	1.4	0.43	0.29	1.5	het.c. 1238G>A het.c. 1304G>A	Arg353Gln Gly375Glu
9	M/21	1	1.4	1.4	0.34	0.28	1.2	het.c. 1061C>T homo.c. 1238G>A	Ala294Val Arg353Gln
10	M/61	-1	1.6	1.5	0.32	0.26	1.2	het.c. 1061C>T homo.c. 1238G>A	Ala294Val Arg353Gln

TABLE 1. *Characteristics of the patients included in the study.*

a *F: female, M: male.*

b *Age at the time of the blood sampling/inclusion in the study.*

c *Estimated according to references [166, 167] http://www.skane.se/upload/Webbplatser/UMAS/SCORE_20090312.pdf (Swedish).*

d, f *Measured using human recombinant thromboplastin.*

e, g *Measured using rabbit brain thromboplastin.*

* *The patient was treated with warfarin at the time of the blood sampling for DNA analysis. The FVII activity values and the PT-INR (Owren method) refer to the values measured prior to initiation of treatment.*

n/a *Not available.*

5.7 Figures

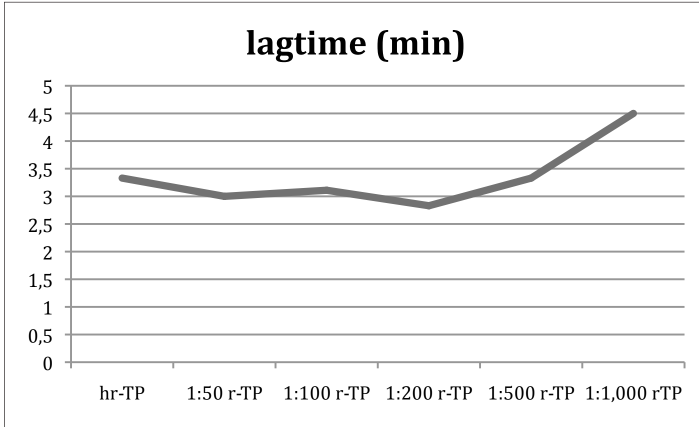

FIGURE 1. *Lagtime (in minutes) measured in the presence of different concentrations of r-thromboplastin in patients with FVII deficiency.*

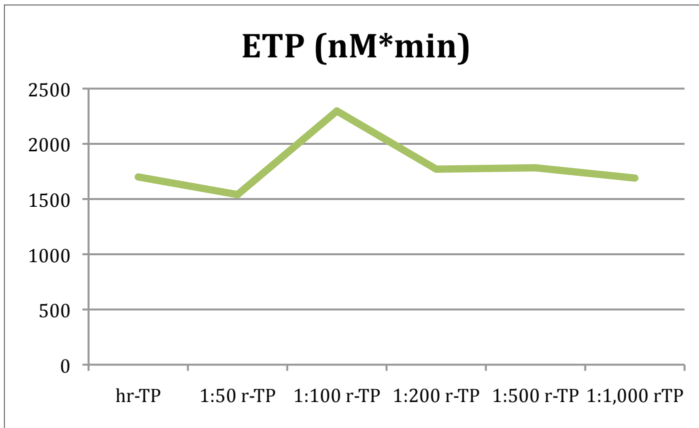

FIGURE 2. *ETP (in nM thrombin*min) measured in the presence of different concentrations of r-thromboplastin in patients with FVII deficiency.*

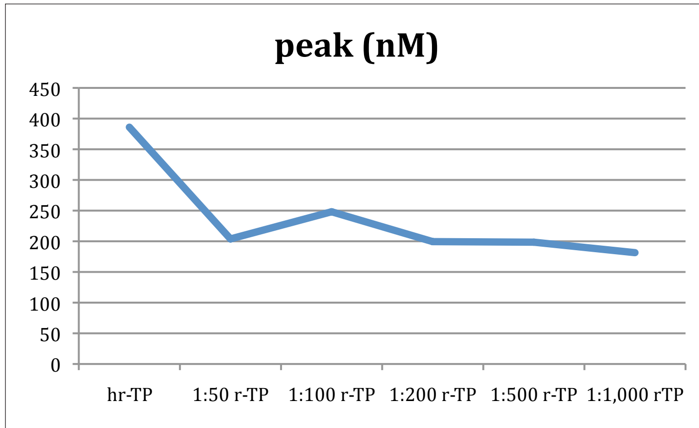

FIGURE 3. *Peak (in nM thrombin) measured in the presence of different concentrations of r-thromboplastin in patients with FVII deficiency.*

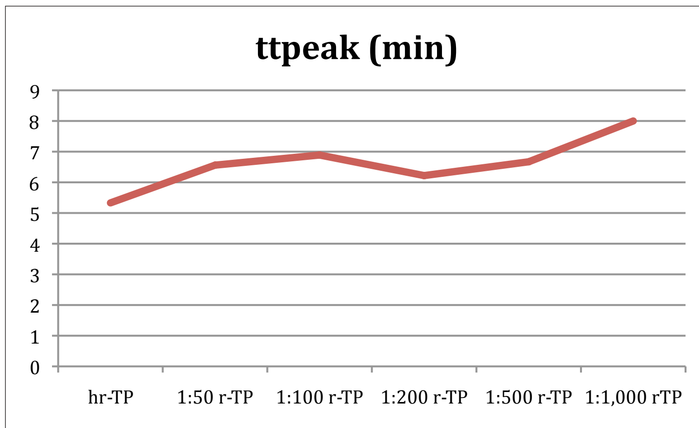

FIGURE 4. *Time to peak (in minutes) measured in the presence of different concentrations of r-thromboplastin in patients with FVII deficiency.*

6 Conclusion and future perspectives

Thrombin generation assays are global haemostatic methods that provide data on the overall haemostatic potential and not just on the effect of isolated factors. Thus, they allow for extensive and thorough coagulation studies in both health and disease, as illustrated in this thesis. The potential applications are unlimited and the role of thrombin generation assays in both clinical praxis and research is increasingly prominent.

One major issue that has to be addressed is standardization. The lack of a globally established reference range limits the usefulness of those assays in patient care. Additionally, the effect of preanalytical variables on the assay might be significant, and has to be taken into consideration when interpreting the results.

Future studies have to focus not only on expanding the applications, but also on establishing robustly designed recommendations on obtaining and handling the samples and on performing the assays. A standardized thrombin generation assay does not have to be limited to the research laboratory. It has the potential to become a crucial tool in diagnostics and in evaluation of treatment.

7 References

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Papers

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