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## Evaluation of global coagulation tests and their implications in haemophilia

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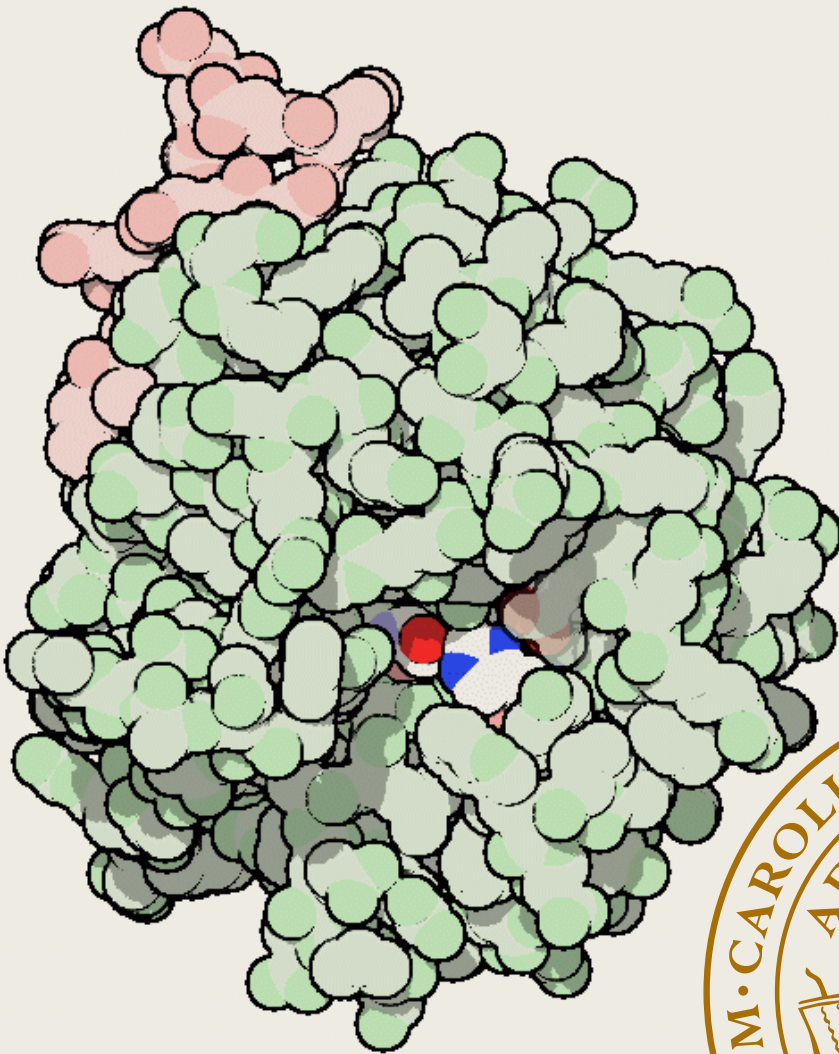
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# Evaluation of global coagulation tests and their implications in haemophilia

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FACULTY OF MEDICINE | LUND UNIVERSITY



Evaluation of global coagulation tests and their implications in haemophilia



# Evaluation of global coagulation tests and their implications in haemophilia

Marcus Ljungkvist



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DOCTORAL DISSERTATION

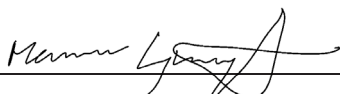
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To be defended at 09.00 on 9<sup>th</sup> May, 2018 in Lilla Aulan, Mediciskt  
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Title and subtitle Evaluation of global coagulation tests and their implications in haemophilia			
<p>Abstract</p> <p>Thrombin generation tests (TGTs) are considered to better reflect the overall haemostatic capacity of an individual than fibrinolytic based assays used in routine coagulation laboratories. The TGT, thrombin generation assay-calibrated thrombogram (TGA-CAT), has in numerous studies proven its usefulness in multiple applications assessing both bleeding and thrombosis disorders. However, a majority of these studies are single-center based and their results needs to be repeated in multi-center studies to gain validity but it has been difficult to achieve due to a low level of standardization for the method. The overall aim with the thesis have been to improve the level of standardization for the TGA-CAT method, comparing it with other TGTs and to evaluate the global coagulation capacity in different cohorts of persons with haemophilia (PWH) with the TGA-CAT method and other TGTs.</p> <p>In paper I, we wanted to know which of two TGT, TGA-CAT and INNOVANCE ETP, that correlate best to FVIII activity(FVIII:C) in persons with haemophilia A(PWHA) and the inter-laboratory variability of the TGA-CAT method. We found that both TGT methods correlated in an equal manner to FVIII:C, <math>r=0.734</math> and <math>r=0.701</math> respectively. The variability of the TGA-CAT method between the two center showed a poor level of agreement. In paper II, we investigate the level of agreement between TGA-CAT results obtained in fresh platelet-rich plasma (f-PRP) and frozen-thawed platelet-rich plasma(ft-PRP) as a previous report had concluded there was no significant difference between the two groups. We assessed our data in a Bland-Altman plot and found a low level of agreement between f-PRP and ft-PRP. Paper III addresses the low level of standardization associated with the TGA-CAT method by evaluating a detailed standardization protocol and the ability of three normal plasma to normalize results. This Nordic multicenter study produced results as robust as standard coagulation assays used in routine laboratories. The objective with paper IV was to assess the effect of physical exercise on specific and global coagulation parameters in a small group of persons with severe HA with age-matched healthy control persons (HS). We also examined if increased levels of VWF after exercise had the ability to prolong the effect of factor concentrate administered just before exercise. We found that maximal physical exercise induced increase in global coagulation capacity in HS, whereas no change was found in persons with severe HA. Our findings do not support the presence of a FVIII-independent mechanism that increases global coagulation, but rather underscores the importance of FVIII in mediating the increased coagulation capacity seen after exercise.</p> <p>To conclude, the results of our investigations have moved the TGA-CAT method one step further to where the test would be feasible to use in large multi-center clinical studies is a reality.</p>			
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# Evaluation of global coagulation tests and their implications in haemophilia

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Lund University, Malmö, Sweden

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*Dedicated to my dear family*

Look up at the stars and not down at your feet.  
Try to make sense of what you see, and wonder about  
what makes the universe exist. Be curious.

- Stephen Hawking

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## List of papers

This thesis is based on the following papers which are appended at the end of the thesis. The papers will be referred in the text by their roman numbers.

- I. **Correlation to FVIII:C in two thrombin generation tests: TGA-CAT and INNOVANCE ETP.** Ljungkvist M, Berndtsson M, Holmström M, Mikovic D, Elezovic I, Antovic JP, Zetterberg E, Berntorp E. *Mediterr J Hematol Infect Dis* 2017, 9(1): e2017064.
- II. **Low agreement between fresh and frozen-thawed platelet-rich plasma in the calibrated automated thrombogram assay.** Ljungkvist M, Lövdahl S, Zetterberg E, Berntorp E. *Haemophilia* 2017;23 (3):e214-e8.
- III. **Evaluation of a standardized protocol for thrombin generation using the calibrated automated thrombogram: A Nordic study.** Ljungkvist M, Strandberg K, Berntorp E, Chaireti R, Holme PA, Larsen OH, Lassila R, Zetterberg E. Submitted to *Haemophilia*.
- IV. **Coagulation factor VIII is vital for increasing global coagulation after exercise.** Ljungkvist M, Olofsson H, Funding E, Berntorp E, Zetterberg E. Submitted to *Haemophilia*.

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# Abbreviations

APC	activated protein C
APTT	activated partial thromboplastin time
AUC	area under the curve
AT	antithrombin
BS	biomedical scientist
CI	confidence interval
CWA	clot waveform analysis
DIC	disseminated intravascular coagulation
EC	endothelial cell
ETP	endogenous thrombin potential
FII	factor II - prothrombin
FIIa	activated factor II - thrombin
FV	factor V
FVa	activated factor V
FVII	factor VII
FVIIa	activated factor VII
FVIII	factor VIII
FVIII:C	factor VIII activity
FVIIIa	activated FVIII
FIX:C	factor IX activity
FIX	factor IX
FIXa	activated factor IX
FX	factor X
FXa	activated factor X
FXI	factor XI
FXIa	activated factor XI
f-PPP	frozen platelet-poor plasma
f-PRP	fresh platelet-rich plasma
ft-PRP	frozen-thawed platelet-rich plasma
GPIb	glycoprotein Ib
GPIIb/IIIa	glycoprotein IIb/IIIa
GPVI	glycoprotein VI
HA	haemophilia A
HB	haemophilia B
HS	healthy control subject

INR	international normalized ratio
MCTH	Malmö Centre of Thrombosis and Haemostasis
PC	protein C
PK	pharmacokinetics
PS	protein S
POC	point of care
PPP	platelet-poor plasma
PWH	person/s with haemophilia
PWHA	person/s with haemophilia A
PWHB	person/s with haemophilia B
ROTEM	rotational thromboelastometry
RP	reference plasma
SD	standard deviation
TF	tissue factor
TEG	thromboelastography
TEM	thromboelastometry
TFPI	tissue factor pathway inhibitor
TGA-CAT	thrombin generation assay-calibrated automated thrombogram
TGT	thrombin generation test
TM	thrombomodulin
t-PA:Ag	tissue type plasminogen activator antigen
VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen





# Introduction

## Clotting mechanisms

### **Haemostasis**

Haemostasis is a protective mechanism that balances the procoagulant, anticoagulant and fibrinolytic activities of the blood flow. It protects against blood loss in case of vascular damage and keeps fluidity of blood in the vessel system. The haemostasis process can be divided into two phases, a primary where the damaged vessel constricts slowing the blood flow and forming a temporary plug from activated and aggregated platelets and a secondary (plasma coagulation) almost simultaneously initiated activating coagulant plasma proteins that stabilises the platelet plug into a fibrin clot. The anticoagulant pathway, involving inhibitors such as AT, PS and APC, ensures a balanced procoagulant process and restricts it to the site where it is needed. The thrombin formed at the end of coagulation stimulates the fibrinolysis process that aims to dissolve the fibrin clot (1).

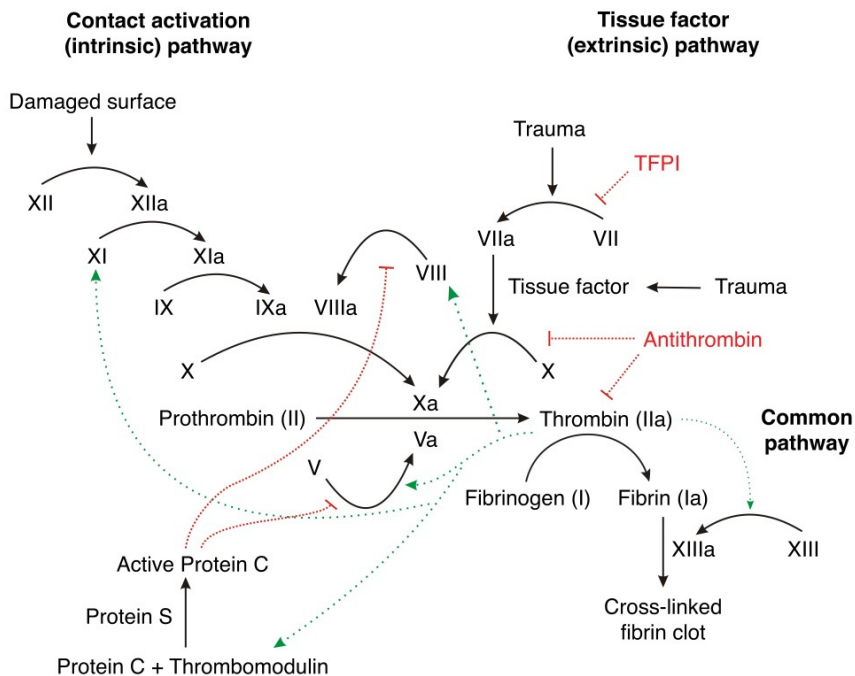
### **Primary haemostasis**

The inside vessel wall is covered by a layer of endothelial cells where blood flow maintaining coagulation inhibitors, thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI) are expressed. When the endothelial lining of a vessel is disrupted due to injury, local vasoconstriction slows blood flow and platelets can adhere at the site of the damaged vessel wall. Platelet receptors glycoprotein Ib (GPIb) and glycoprotein (GPVI) bind with von Willebrand factor (VWF) and collagen, respectively, to anchor to the sub-endothelium (2, 3). The activation that follows renders the platelets a morphological change, a release of active substances from granules and an expression of the fibrinogen receptor glycoprotein IIIbIIa (GPIIbIIa) (4). Arriving platelets can now aggregate via inter-platelet fibrinogen bridges and a plug is formed (5, 6).

## Plasma coagulation

### Coagulation cascade

The coagulation process can be described as a cascade in which a number of proteins, or enzyme, activate in sequence the next protein, proenzyme, and add to the reaction step by step to its final product, a strong fibrin clot. The coagulation cascade can be subdivided into an intrinsic and an extrinsic pathway converging in a common pathway where factor X is activated, FXa that in turn convert prothrombin to thrombin (7, 8)(Figure 1). The contact-activated intrinsic pathway affect the *in vitro* process and therefore only have a limited role in the *in vivo* or trauma-initiated haemostasis. However, one of the roles of the pathway is to act as an initiator of coagulation and to keep the positive feedback loop of continuing coagulation ongoing. The extrinsic pathway is triggered by injury-exposed sub-endothelium that activates TF and FVII to its activated form FVIIa which, in turn, activate FX and the cascade propagates (9). The common pathway commences with FXa and its co-factor FVa, the prothrombinase complex, cleaving prothrombin to thrombin that converts fibrinogen into the end product of fibrin.



**Figure 1** Cascade model of the haemostasis. The roman numerals indicate inactive and active coagulation factors (see Abbreviations).

### *Cell-based model of coagulation*

A little over a decade ago a new way of looking at the coagulation process was introduced. Instead of describing the process as a cascade the cell-based coagulation model describes the coagulation process as overlapping stages where the coagulation factors gather and assemble on cell surfaces (10, 11). In this model, three phases are described, the initiation phase, the amplification phase and the propagation phase (Figure 2). The first step, the initiation phase starts when vascular injury occur on TF-bearing cells where TF activates FVII which in turn activates small amounts of FIX and FX. FXa binds to FVa and converts a small amount of prothrombin to thrombin initiating coagulation.

The second step, the amplification phase occurs on the surface of activated platelets, Platelets that have adhered to the site of the injury are activated by the small amounts of thrombin that have been generated in the initiation phase. The generated amounts of thrombin also cleaves FV, FVIII and FXI into their activated forms.

The third step, the propagation phase also occurs on the surface of activated platelets. As soon as the tenase complex (FIXa/FVIIIa) has formed on the platelet surface a rapid generation of FXa starts. FXa binds to FVa to form the prothrombinase complex. This complex cleaves prothrombin into thrombin resulting in a burst of thrombin generation which in turn leads to the cleavage of fibrinogen into fibrin. Thrombin also activates FXIII, which strengthens the fibrin clot (12).

There are three major inhibitor pathways preventing uncontrolled clot formation. The TFPI inhibits TF-FVIIa when the coagulation has been initiated (13, 14). Antithrombin inhibits thrombin, TF-FVIIa complex, FIXa, FXa and FXI (15). APC inactivates FVa and FVIIIa and the inactivating capacity is greatly enhanced when binding to its circulating co-factor PS (16).



# Laboratory assessments

## Thromboelastometry

Initially described in 1948 by Hartert (18) thromboelastography (TEG) provides a real-time assessment of viscoelastic clot strength in whole blood. Rotational thromboelastometry (ROTEM) evolved from TEG technology and both devices effectively measure the capacity of the coagulation process in terms of maximal fibrin clot formation (19). The main differences between TEG and ROTEM is the detector system and the way movement is initiated to the sample, a torsion wire/from the cup and an optical detector/from the pin, respectively. The preference for which viscoelastic tests to use appears to reside primarily on geography, with centers in North America favouring TEG while Europeans prefer ROTEM. It is difficult to directly compare the tests due to differences in the activators utilized in each device. (20). Overall, the prevalent opinion is that the two tests are equivalent with interchangeable results and interpretations. These point of care devices (POC) allow visual assessment of blood coagulation from clot formation, through propagation, and stabilization, until clot dissolution. Computer analysis of the output allows sophisticated clot formation/dissolution kinetics and clot strength data to be generated. Activation of clot formation can be initiated with both intrinsic (kaolin, ellagic acid) and extrinsic (tissue factor) activators. In addition, the independent contributions of platelets and fibrinogen to final clot strength can be assessed using added platelet inhibitors (21). The method is suitable for detecting and treating coagulopathy in trauma care, cardiac surgery and liver transplantation, particularly in patients where fibrinogen levels are low (22-24). Some evidence suggests these algorithms might reduce transfusions (25, 26), but further study is needed to assess patient outcomes. Although the method is able to detect hypercoagulable situations it is not frequently used to predict thrombosis but rather to tailor antithrombotic therapy (27). The method is commonly utilized as a complement to conventional haemostasis screening plasma based analyses.

Blood (300 µl, anticoagulated with citrate) is placed into the disposable cuvette using an electronic pipette. A disposable pin is mounted to a shaft which is connected with a thin spring (the equivalent to Hartert's torsion wire in TEG) and slowly oscillates back and forth. The signal of the pin suspended in the blood sample is transmitted via an optical detector system. TEM is started by adding appropriate reagents. The instrument measures and graphically displays the changes in elasticity at all stages of the developing and resolving clot. The typical test temperature is 37 C, but different temperatures can be selected, e.g. for patients with hypothermia. In contrast to TEG with its pendulum-like principle, the design of the TEM

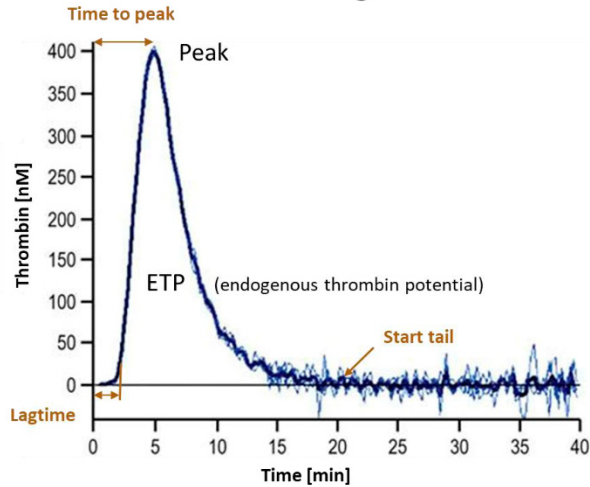
viscoelastic detection system makes it quite robust and insensitive against mechanical shocks or vibrations.

### **Thrombin generation assay-calibrated automated thrombogram**

In the early 1950s the first version of thrombin generation was described by two British groups (28, 29). The method had a complicated protocol and was not “operator-friendly” with its time-consuming continuous sub sampling procedure. Since the 1980s Hemker *et al.* have developed the method in different steps to the CAT<sup>®</sup> assay we have today (30, 31). The method is considered to reflect the global coagulation capacity in vitro (32, 33). To enable a continuous measurement of the thrombin generation a slow-acting substrate has been developed. The fluorogenic substrate is unaffected by turbidity caused by clot formation and the presence of platelets. With chromogenic methods platelets and fibrinogen needs to be removed from the sample prior to measurement (34). There is a non-linear relationship between thrombin activity and the fluorescent signal due to the inner filter effect and substrate consumption of fluorescence measurements. This problem is overcome by comparing the fluorescent signal to a constant thrombin calibrator with a known thrombin activity in a non-clotting plasma sample (30).

The measurements take place in a 96-well round bottom plate where 20 µl trigger reagent is pipetted in one well and 20 µl Thrombin Calibrator in another well. Then 80 µl of plasma added to each of the two wells. This pairing is recommended to be made in triplicate or at least in duplicate (34). All pipetting is advised to be carried out with reverse pipetting to avoid introducing air bubbles in the wells (affecting the measurement). Then the plate is put in the fluorometer, and 10 minute 37°C incubation commence. Then a mix of Fluo-Substrate and the pre-heated Fluo-Buffer (37°C waterbath) is made and added to the analyzer. After the incubation period the dispensing system is prepared with the substrate, FluCa solution, and the analysis is started. The system reads the data and display the thrombin concentration in time on a screen. The measurements are approximate during the measurement duration and will be further analyzed the moment the measurements stop. The results are presented in a thrombogram from which the different parameters are calculated, lagtime, time to peak (tpeak), starttail, peak thrombin concentration, endogenous thrombin potential (ETP) (Figure 3). Peak and ETP values are often the main parameters referenced to in clinical studies(35-37).

## Thrombogram



**Figure 3** Parameters of the thrombogram: lagtime (min), time to peak (min), peak (nM thrombin), endogenous thrombin potential (ETP)(nM/min), starttail (min).

## Clot wave formation

The method, clot waveform analysis (CWA), is based on a technique reported by Braun and his co-workers who assessed activated partial thromboplastin time (APTT) and prothrombin time (PT) with light transmission (38). The optical detection system recognizes the clot formation process by measuring changes in transmittance, or absorbance, of a light beam through the analysed sample. The transmittance or absorbance is continuously recorded over time. The graph is computerized and the first and second derivatives are added to the final plot. The resultant curves obtained are called “waveform” because of their sigmoidal profile (38). The tracing against the time should reflect the whole process of clot formation and clot lysis. The qualitative examination of the clot formation curve and the multiple quantitative parameters provided by CWA can give valuable information in addition to clotting time obtained by routine assays. CWA is mainly based on the aPTT assay; however, PT (INR) or modified assays have also been used in various studies. At present, HA management and early diagnosis and prognosis of disseminated intravascular coagulation (DIC) (39) are the two main clinical fields in which CWA is designed to deliver improvements to patient management. However, many other studies have published on further potential clinical applications such as lupus anticoagulant diagnosis and anticoagulant monitoring. The method is considered to be inexpensive, rapid and is readily available on

coagulation analyzers with optical detection systems. Nevertheless, as studies are limited to small cohorts, extensive prospective clinical trials are mandatory to make the leap to clinical application. Efforts have been made to standardize the assay and the use of specific reagents, which do not interfere with light transmission/absorption seems to be one important issue (40).

## **Specific coagulation factor measurement**

### *One-stage FVIII:C Assay*

FVIII concentration in plasma is determined by evaluating the ability of the test plasma to correct the coagulation defect of FVIII-free plasma (<0.01 kIE/L). A test base is produced from FVIII-depleted plasma and a standard reference plasma of known concentrations by a serial dilution of the two, yielding a standard curve. A minimum of three dilutions of test plasma in FVIII-depleted plasma is analyzed and the clotting times received are compared with the standard. The FVIII:C in the test plasma is given in kIU/L (kilo international units/liter), if the method is calibrated with an international standard.

### *Chromogenic Two-stage FVIII:C Assay*

The two-staged FVIII:C assay is based on the assumption that the amount of FXa generated is proportional to the FVIII activity in the sample. FX is first activated by FVIIIa and FIXa, and the FXa activity is determined in the second step. Today, the assay is performed using the chromogenic substrate S-2222-pNA, and the FXa activity is determined by hydrolysis of the chromogenic peptide in which the chromophoric pNA is liberated. The colour intensity is read photometrically at 405 nm, and is proportional to FVIII:C.

One-stage is the method of choice in North America and the more common method world-wide, but the two-stage or chromogenic FVIII:C method is increasingly used especially in Europe. The One-stage is known to be the cheaper of the two methods, a fact argued by some saying the difference in cost can be eradicated with optimized reagent use in the Two-stage method. Heparin and Lupus anticoagulant cause falsely low FVIII:C values in the One-stage clot method.

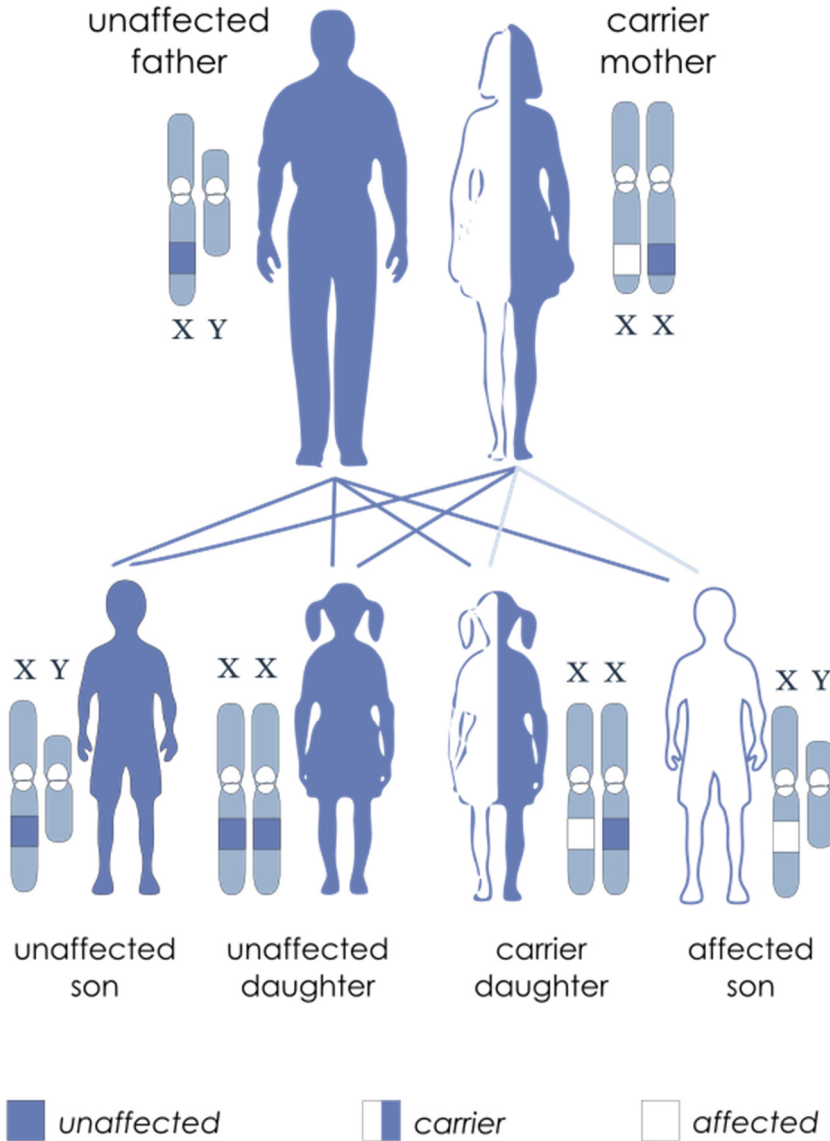
## **Haemophilia**

The word haemophilia comes from the Greek *haima* meaning blood and *philia* meaning love – love of blood (41). Haemophilia is a rare congenital bleeding disorder affecting mainly males since it is inherited recessively via the X-



chromosome of female carriers (Figure 4). The disease is caused by the deficiency or absence of either clotting protein factor VIII (haemophilia A (HA)) or factor IX (haemophilia B (HB)) in plasma. Female carriers may in some rare cases have FVIII/FIX plasma levels in the range of haemophilia. The ratio between HA and HB is roughly 4:1 in all ethnic groups (42). Haemophilia is divided into 3 groups based on plasma activity levels of FVIII(HA) and FIX(HB), respectively: severe ( $<0.01$  IU/ml), moderate (0.01-0.05 IU/ml) and mild ( $>0.05$ - $<0.40$  IU/ml)(43). Normal range is set to be between 0.50-1.50 IU/ml. Signs and symptoms of hemophilia vary, depending on your level of clotting factors. Haemorrhagic episodes may occur anywhere in the body. Joint bleeds account for 70% to 80% while muscle bleeds account for 20% to 30% of the bleeding episodes. Repeated bleeding manifestations in joints are predominantly found in severe and moderate haemophilia, if absence of adequate clotting factor replacement, and often lead to various degrees of arthropathy. In severe haemophilia, where factor level is below 1% of normal, joint and muscle bleeding, as well as internal bleeding, may occur spontaneously or after minor trauma. In moderate haemophilia, factor level is higher, 1-5% of normal, but still may cause spontaneous bleeds or bleed after minor trauma. In mild haemophilia, where factor level is more than 5% and up to 40% of normal, spontaneous bleeding is rare but may occur after minor trauma (43, 44). The percentage distribution between the severity groups of HA/HB in the western countries are about 46%/34% severe, 15%/32% moderate and 39%/33% mild (45). Figures may vary between countries due to the standard of haemophilia care and diagnostic capacity accessible, as mild haemophilia may be underdiagnosed in low economy countries. Haemophilia A and B are comparable in heredity and are commonly considered to be very hard to discriminate between clinically, although recent studies indicate a more benign course of HB (46, 47).

# X-linked recessive inheritance



**Figure 4** Inheritance patterns of haemophilia. With an unaffected father and a carrier mother there is a 50% chance at each birth that a son will have haemophilia. There is a 50% chance at each birth that a daughter will carry the haemophilia gene.

## Physical activity in persons with haemophilia

There is a high risk of spontaneous joint bleeds among persons with severe HA that might lead to disabling arthropathy unless prevented by prophylactic treatment with factor concentrate starting at an early age (48). Persons with severe HA who receive prophylactic treatment since early childhood suffer few or no joint bleeds and are able to participate in everyday activities including different kinds of sport (49). There are several benefits with physical activity for PWH, improved proprioception, strength of joint-stabilizing muscles that reduce the risk of joint bleeds and an improved social wellbeing (50). However, in high energy sports such as soccer, rugby and wrestling, the risk of bleeding due to trauma is considered to outweigh the benefits of exercise among persons with severe HA (51). Physical activities considered relevant in the management of haemophilia are aerobic exercise, strengthening of specific muscles, joint mobility and muscle stretching exercise, exercises designed to improve balance and proprioception, weight-bearing exercises, and exercise in water (hydrotherapy). There are many studies conducted with the intention of investigating possible benefits with different kinds of exercise and intensity on different cohorts of PWH. In a Cochrane report from 2016 the aim was to systematically review the available evidence on the safety and effectiveness of exercise for people with haemophilia (52). The authors found it difficult to pool the results due to the heterogeneity of the interventions and the outcome measures and therefore compromising their results. Due to low number of subjects and potential bias all but one study had their outcomes rated as low or very low in a quality of evidence and results should therefore be interpreted with caution. However, for pain relief in adults, hydrotherapy may be more effective than land exercises. When the aim is set to improving muscle strength, functional exercises such as treadmill walking and partial weight-bearing exercises seem to be more effective than static or short arc exercises. None of the investigated studies reported any adverse effects, changes in bleeding frequency or bleeding due to the exercise interventions used. In a study where boys with HA and HB of different severities participated in organized sport activities, it was shown that the number of injuries and bleeds were the same in both cohorts (53). However, assessing any type of exercise interventions on persons with severe haemophilia should be made with utmost caution.

## Effect of physical activity on specific and global coagulation parameters

Global coagulation assays, such as TGA-CAT and ROTEM, provides a measure of the total coagulation capacity of a person. These assays are considered to better represent the bleeding tendency than the measurement of specific coagulation factors, as they have been shown to better correlate with the differences in clinical severity of HA observed in patients with the same FVIII levels (54, 55). Samples taken in a group of healthy persons before and after finishing a marathon run yielded unclear ROTEM results, some indicating an increased coagulation capacity while others showed the opposite (56). There is some evidence, however conflicting, that the global function of the coagulation actually increases during exercise in healthy subjects (57). This increase is thought to be mediated by a well-known exercise-induced elevation of FVIII and VWF levels (58-62). Raised levels of VWF, carrier protein of FVIII, prolongs the half-life of the latter (63). Increased FVIII and VWF levels in response to exercise have been shown in patients with mild (64-67) and moderate (65) HA. However, no increase in FVIII levels among persons with severe HA after submaximal exercise was recorded in a study by Koch *et al.* (66). Similar results were obtained in a paediatric cohort with haemophilia, where increased FVIII:C, VWF:Ag and VWF activity was seen in the mild-moderate group and no change in FVIII:C in the severe group after exercise (68). A recent study concludes that the current available *ex vivo* and *in vivo* evidence suggests that endogenous FVIII is released by ECs from different vascular beds in response to epinephrine following strenuous exercise in patients with non-severe haemophilia (69). The FVIII and VWF exercise-induced increases seen in both healthy control persons and PWH is not accompanied with increased levels of FIX. The unaffected levels of FIX would render PWHB with no possibility of a potential improvement of their coagulation capability and the benefits from physical exercise would stay with improved physical and social wellbeing. However, recently a study (70) speculated in the fact that the exercise induced increased levels of FVIII and VWF after exercise would enhance the enzymatic function of FIX, as FVIII is the cofactor to FIX, and thereby possibly improving the overall haemostasis.

# Aims of the thesis

The overall aim was to evaluate global coagulation tests and their implications in haemophilia with special focus on the TGA-CAT method and its standardization issues.

## **Paper I:**

Which of two TGTs, TGA-CAT or INNOVANCE ETP, correlate best with the FVIII activity level in a group PWH?

To investigate the inter-laboratory variability of the TGA-CAT method between two center.

## **Paper II:**

To investigate the level of agreement between f-PRP and ft-PRP ETP and peak results in the TGA-CAT method.

## **Paper III:**

To further improve the level of standardization of the TGA-CAT method by evaluating the use of a detailed standardization protocol and to test the ability of a number of reference plasma to normalize results with the aim to reduce the inter-laboratory variability.

## **Paper IV:**

To investigate the exercise-induced effect on global coagulation and specific coagulation factors in persons with severe HA.

To investigate if exercise-induced raise of VWF extend the half-life of pre-exercise administrated FVIII-concentrate in persons with severe HA.



# Material and methods

## Study populations

The first study (Paper I) was approved by the Ethics Committee, Stockholm (Dnr. 01-0003; 2006/778-32; 2013/263-32). The rest of the studies were approved by the Regional Research Ethics Committee at Lund University (Dnr. 2012/373(Paper II); 2016/451(Paper III); 2015/729(Paper IV)). Informed consent was obtained from all participants prior the studies.

### **Paper I**

Two haemophilia care center provided the study material: The Hematology Center, Karolinska University Hospital, Stockholm, Sweden and the Hemophilia Centre, Belgrade, Serbia. Stockholm. All three disease severity levels of HA where represented in the study material, mild, moderate and severe. The Stockholm samples were collected from 23 subjects (10 with mild, five with moderate and eight with severe HA) and the Belgrade were collected from 17 subjects (six with mild, five with moderate and six with severe HA).

### **Paper II**

Blood samples were collected from 41 PWH treated at the Malmö Centre for Thrombosis and Haemostasis. Thirty-three had severe HA, six had severe HB, one had moderate HA and one had mild HB. Blood samples were also collected from 45 HS.

### **Paper III**

Three different pools of plasma were used in the study (*hypo*-, normal- and *hyper*-coaguable plasma). The *hypo*- plasma pool consisted of four persons with severe HA treated at the Malmö Centre for Thrombosis and Haemostasis (MCTH). The normal plasma pool consisted of four healthy volunteers all having ETP values close to the mean of the TGA-CAT normal range material established at MCTH. The *hyper*- plasma pool consisted of three healthy volunteers all having ETP values of >2 standard deviations (SD) of the mean from the same TGA-CAT normal range material as above. The FVIII:C (kIE/L) in the pooled plasma groups was 0.024 for *hypo*-, 0.55 for normal and 0.95 for *hyper*-.

## **Paper IV**

Ten male subjects with severe HA were included alongside 10 age-matched HS. The exclusion criterias were chronic disease, and ongoing heart or lung disease requiring pharmacological treatment, as well as any musculoskeletal disability that could affect the performance of a maximal exercise test on a bicycle ergometer.

### **Blood sampling and plasma preparation**

For details about blood sampling and plasma preparations, PPP and PRP, see the corresponding section of each paper.

## **Methods**

### **Thrombin generation assay-calibrated automated thrombogram (Papers I-IV)**

Thrombin generation measurement was conducted according to the method described by Hemker *et al.* (30), for details see the corresponding section in each paper. However, in paper III we did not use the manual of the manufacturer. We used a detailed standardization laboratory protocol developed at MCTH and finalized after implementing valuable operator feedback received during the pre-study phase of the study (Appendix A). The standardization laboratory protocol is based on the manual of the manufacturer, though more detailed and built up around a 50 minute countdown. It includes comments and pictures showing important steps of the procedure. There is also a pre-assay checklist, microplate pipetting scheme and a test run log among the standardization protocols (Appendix B). All TGA-CAT measurements from MCTH were performed by the author. TGA-CAT measurements from Stockholm (paper I) were performed by Maria Berndtsson, MD.

### **INNOVANCE ETP (Paper I)**

For details see the corresponding section in paper I. All INNOVANCE ETP results were analyzed in Stockholm by Nida Mahmoud Hourani Soutari, BS.



## **Thromboelastometry (ROTEM)(Paper IV)**

For details see the corresponding section in paper IV. ROTEM (rotational thromboelastometry) results were analysed by BS at the Malmö coagulation laboratory.

## **Laboratory analyses**

In paper IV, the following analysis were performed in the control group: von Willebrand factor antigen (VWF:Ag), von Willebrand factor activity (VWF activity), international normalized ratio (INR), activated partial thromboplastin time (APTT), Protein C (PC), Protein S (PS), antithrombin (AT), tissue plasminogen activator antigen (t-PA:Ag), fibrinogen and coagulation factors II, V, VII, VIII, IX, X, XI coagulant activity were analyzed. In the patient group, only TGA-CAT, ROTEM, VWF:Ag, VWF activity, FVIII:C and t-PA:Ag were analyzed. This limitation measure was taken to avoid substantial blood loss in PWhA subjected to maximal physical exercise. All coagulation factor analyses were performed according to routine protocols at the department of Laboratory Medicine, SUS, Malmö, Sweden (accredited by SWEDAC).

## **Pharmacokinetics (Paper IV)**

In paper IV, individual pharmacokinetic (PK) parameters were determined following factor VIII administration, without and in conjunction with physical exercise, by Bayesian estimation (71). The estimation employed a previously published population PK model (72) and was based on information from the observed factor VIII activities, the subject's body weight and age, and whether the concentrate was a plasma or recombinant derived product. The estimation was performed in NONMEM 7.3 (73). Based on the parameters, the terminal half-life and AUC were calculated. The pharmacokinetic calculations were performed by Siv Jönsson, PhD, Sr. Researcher and Elisabet Bielsen, PhD, Asst. Professor at Farmbio, Uppsala University.

## **Statistical methods**

Agreement statistics (paper I-III) was applied by using Bland-Altman plots to determine the level of agreement of assessed data. Due to lack of normal distributed data the use of confidence intervals (CI) was not possible. Spearman's (non-parametric) rank correlation test was used to determine the associations between

parameters (paper I). In paper IV the Wilcoxon signed-rank test was used to demonstrate differences between patients and controls. For the analyses where samples from more than two time points were analyzed, the Wilcoxon signed-rank test was run as a post hoc analysis for the variables where the Friedman test reported statistically significant changes over time ( $p\text{-value}<0.05$ ). The Bonferroni correction was applied to adjust for multiple comparisons with a significance level of  $p\text{-value}<0.025$ . In paper III, the mean, standard deviation (SD) and coefficient of variation (CV)(%) were calculated for TG parameters and the exclusion of outliers was made with the outlier labeling method (74, 75). The Wilcoxon signed-rank test was used to determine the correlation between the analysing temperature and RT of the participating center. Statistical analyses were performed by the author of this thesis using IBM SPSS for Windows, version 22-24 (Armonk, NY: IBM Corp. USA).

## Results

### Thrombin generation correlation to FVIII:C

In paper I we evaluated which of two TGTs that showed the best correlation to FVIII:C in a cohort of PWhA consisting of mild, moderate and severe disease severity. The TGA-CAT and INNOVANCE ETP parameter reflecting the total amount of thrombin produced during the assay, ETP and AUC respectively, correlated in an equal manner to FVIII:C. The level of correlation,  $r=0.734$ (ETP) and  $r=0.701$ (AUC), was not improved when correlation measurements were assessed on disease severity level of the cohort (Table 1). When comparing the TGA-CAT parameter ETP with the corresponding parameter for INNOVANCE ETP AUC a correlation of  $r=0.546$  was found.

**Table 1.**

Correlation coefficient (r) for ETP and AUC to FVIII:C in all patients and grouped by disease severity.

	FVIII:C			
	Severe	Moderate	Mild	All patients
TGA-CAT - ETP	0,240	0,172	0,458	0,734
INNOVANCE ETP - AUC	0,185	0,305	0,238	0,701

FVIII:C; FVIII activity, ETP; endogenous thrombin potential, AUC; area under the curve,

### **Intra- and inter-assay repeatability**

In paper III, the mean intra-assay CV for the six participating laboratories was below 7.3% for all of the *hypo*-, normal or *hyper*- plasmas and TF concentrations tested. Four of the six laboratories had inter-assay CVs of 10% or lower for all parameters.

### **Inter-laboratory reproducibility – variability in the TGA-CAT method**

All five measured TGA-CAT parameters obtained CVs of 10% or lower under all testing conditions except for ETP and peak values in *hypo*- plasma where only the peak value in PPP-reagent had a CV below 10%. The other ETP and Peak values for *hypo*- plasma obtained CVs between 12% and 14% (paper III)(Table 2).

**Table 2.**

Inter-laboratory CV results. Robust mean and associated interlaboratory CVs with 1pM and 5pM triggering conditions. Bold characters indicate CVs  $\leq 10\%$ . Symbols ( $\uparrow = \downarrow$ ) illustrate changes in CVs as compared with raw data. Normalised results are expressed as a percentage of a RP. \* indicates that Siemens Control Plasma P (dli. 1+5) was used for for hypo-plasma and Affinity Protein S deficiency plasma for hyper-plasma for normalization.

Sample type	Parameters	PPP-reagent LOW						PPP-reagent									
		Raw data		Normalized data HemosiL		Normalized data "dedicated"*		Raw data		Normalized data HemosiL		Normalized data "dedicated"*					
		Robust mean	CV (%)	Robust mean (%)	CV (%)	Robust mean (%)	CV (%)	Robust mean	CV (%)	Robust mean (%)	CV (%)	Robust mean (%)	CV (%)				
Hypo	ETP	312.2	12	41	13	$\uparrow$	35	22	$\uparrow$	827.8	13	53	9	$\downarrow$	85	18	$\uparrow$
	Peak	19.8	14	33	15	$\uparrow$	49	21	$\uparrow$	64.9	9	26	6	$\downarrow$	149	9	=
	Lagtime	10.4	<b>8</b>							2.8	<b>8</b>						
	ttPeak	21.3	<b>6</b>							8.8	<b>6</b>						
	Starttail	48.0	<b>4</b>							34.8	<b>4</b>						
Normal	ETP	997.7	9	129	<b>3</b>	$\downarrow$				1271.3	9	81	<b>4</b>	$\downarrow$			
	Peak	109.7	<b>8</b>	180	<b>3</b>	$\downarrow$				185.5	<b>6</b>	73	<b>2</b>	$\downarrow$			
	Lagtime	7.2	<b>7</b>							2.9	<b>8</b>						
	ttPeak	12.8	<b>4</b>							6.7	<b>4</b>						
	Starttail	33.6	<b>3</b>							25.8	<b>4</b>						
Hyper	ETP	1552.6	<b>6</b>	197	<b>6</b>	=	83	<b>5</b>	$\downarrow$	1773.0	<b>8</b>	112	<b>3</b>	$\downarrow$	92	<b>3</b>	$\downarrow$
	Peak	200.0	<b>5</b>	321	<b>10</b>	$\uparrow$	81	<b>6</b>	$\uparrow$	293.2	<b>5</b>	115	<b>5</b>	=	86	<b>4</b>	$\downarrow$
	Lagtime	7.3	<b>5</b>							3.1	<b>8</b>						
	ttPeak	11.6	<b>4</b>							6.2	<b>4</b>						
	Starttail	32.4	<b>3</b>							26.1	<b>4</b>						

ETP; Endogenous Thrombin Potential, ttPeak; time to Peak, CV; coefficient of variation.

## Inter-laboratory reproducibility - agreement analysis in the TGA-CAT method

We decided an acceptable limit of agreement for the TGA-CAT method to be that 95% of the observations would fall within an agreement interval of  $\pm 10\%$  (paper I-III) when plotted in a Bland-Altman diagram.

Agreement analysis results between MCTH and Stockholm was poor, 29% of the observations reached the agreement interval of  $\pm 10\%$  for the ETP parameter (paper I).

In paper II, agreement analysis assessing fresh and frozen-thawed platelet-rich plasma TGA-CAT results in a cohort of PWH and HS at MCTH yielded poor levels of agreement. The parameter showing the highest level of agreement of the whole cohort was ETP with 47%. For results obtained when the cohort is divided into PWH and HS see Table 3.

**Table 3.**

Percentage of observations for ETP and peak in PPP and PRP for All, PWH and HS that are within the agreement limits of  $\pm 10\%$ .

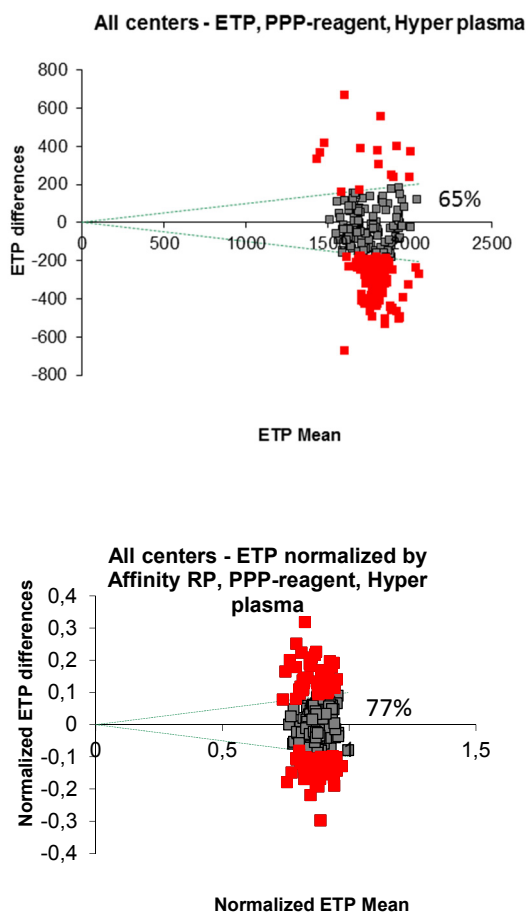
		All n(%)	PWH n(%)	HS n(%)
PRP	ETP	47	29	62
	Peak	7	15	0
PPP	ETP	26	34	18
	Peak	20	22	18

In the TGA-CAT standardization study (paper III) each center was matched pairwise with all other participating centers yielding in total fifteen agreement test groups (Table 4) that were all assessed with agreement testing in Bland-Altman plots with up to three hundred observations in each. The best level of agreement was observed in ETP with PPP-reagent and *hyper*- plasma, 65% and was improved after normalization to 77% and can be seen in Figure 5.

**Table 4.**

Scheme showing agreement analysis pairings.

	Malmö	Aarhus	Gothemburg	Helsinki	Oslo	Stockholm
Malmö	-	X	X	X	X	X
Aarhus	X		X	X	X	X
Gothemburg	X	X		X	X	X
Helsinki	X	X	X		X	X
Oslo	X	X	X	X		X
Stockholm	X	X	X	X	X	



**Figure 5.**

Results from all centers: ETP measurements in *hyper*- plasma analyzed with PPP-reagent were plotted in a Bland-Altman plot. 65% of the observations were within the green dashed cone representing  $\pm 10\%$  level of acceptance. This level was improved to 77% after normalization with Affinity protein S deficiency plasma.

## **Normalization of data effect on TGA-CAT reproducibility results**

Normalization of data using CryoCheck pooled normal plasma (Precision BioLogic, Dartmouth, Canada) increased the percentage of observations within the agreement interval of  $\pm 10\%$  from 29% to 41% for the ETP variable (paper I).

In the standardization study three reference plasmas (RP) ability to normalize result were evaluated, one intended to normalize results over the whole measuring range (HemosIL Calibration Plasma) and two so called dedicated normalization plasmas (Siemens Control Plasma P and Affinity protein S deficiency plasma) intended to normalize the *hypo*- and *hyper*- plasma results, respectively. Normalization with HemosIL reference plasma (RP) resulted in equal or lower CVs for all ETP and peak values except for Hypo ETP/peak and Hyper peak values with the low TF value. Normalization with Affinity RP yielded lower CVs in three of four settings, while normalization with Siemens RP was incapable of improving the CV in any of the four *hypo*- plasma cases.

## **Effects of exercise on coagulation parameters in patients with haemophilia A compared to controls**

### *ROTEM*

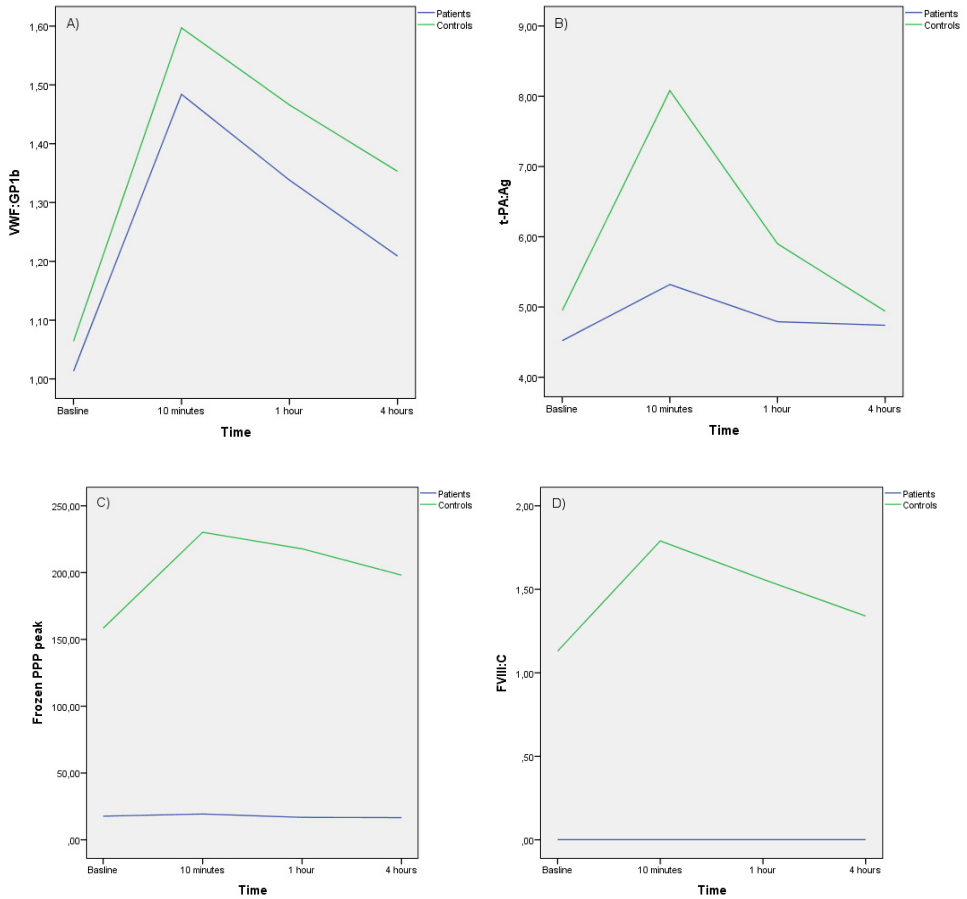
Exercise induced changes in the ROTEM parameters indicating an increased coagulation capacity in the controls, while among persons with severe HA the results displayed no certain effects of the exercise.

### *TGA-CAT*

TGA-CAT parameters were evaluated in three plasma types, fresh and frozen PPP as well as in fresh PRP, before and after maximal physical exercise in controls and in persons with severe HA. Controls displayed significant increases in all peak and ETP measurements except for ETP when analyzing frozen PPP. No statistically significant changes before and after exercise could be seen in any TGA-CAT parameters in the patient group. Peak results can be seen in Figure 6.

### *Coagulation factor analyses*

FVIII:C, VWF and t-PA:Ag all showed exercise induced significant increases in both patients and controls, except for FVIII:C in persons with severe HA where no increase was seen (Figure 6).



**Figure 6**

Analyses of samples taken from patients (blue line) and controls (green line) at baseline, as well as 10 minutes, 1 hour and 4 hours after a maximal exercise test: VWF:GP1b (A), t-PA:Ag (B), frozen PPP peak (C) and FVIII:C (D). The differences between patient and control results were statistically significant (with Bonferroni correction) after 10 minutes and 4 hours for frozen PPP Peak and FVIII:C. A significant difference was also observed for t-PA:Ag after 10 minutes, whereas no statistically significant difference was observed in VWF activity between patients and controls. VWF:GP1b; von Willebrand factor activity, t-PA:Ag; tissue plasminogen activator antigen, FVIII:C; factor VIII activity, frozen PPP peak; frozen platelet poor plasma peak.



## Pharmacokinetics (PK)

None of the six pharmacokinetic variables showed any statistically significant differences between PK parameters obtained when FVIII concentrate was administered before exercise and when FVIII concentrate was given without exercise (Table 5).

**Table 5**

Pharmacokinetic parameters analyzed in patients administered with factor VIII concentrate with and without physical exercise

	Maximal physical exercise		p-value
	Yes	No	
Clearance (CL mL/h)	234 (122-290)	192(148-261)	0.35
Central volume of distribution (V1 mL)	3108 (2378-4702)	3512 (2346-3735)	0.92
Intercompartmental clearance (Q mL/h)	308 (262-342)	308 (262-342)	1.00
Peripheral volume of distribution (V2 mL)	308 (248-354)	308 (248-354)	1.00
Terminal half-life (h)	11.2 (7.5-15.7)	13.4 (10.1-14.7)	0.29
AUC (IU*h/mL)	10.0 (7.4-16.4)	11.0 (10.3-15.6)	0.46

Results are median (range). A p-value is presented for each pharmacokinetic parameter (p-value <0.05 was considered to be significant).



# Discussion

The TGA-CAT method has in numerous studies displayed multiple areas of utility including diagnosis and management of bleeding disorders (36, 76-78), detecting hyper-coagulability (79-81), and monitoring and characterization of oral anticoagulant drugs (82, 83). The key for allowing the findings of these studies to be validated in large prospective clinical multi-center studies is obtaining an adequate level of standardization for the method.

The low standardization level of the TGA-CAT method and how to improve it have been central to this thesis.

The ability of the TGA-CAT method to assess platelet-rich plasma gives the physician a tool to evaluate the platelet impact on the global coagulation capacity of a patient. Platelets are very sensitive and easily get activated. When assessing platelet-rich plasma the pre-analytical handling of the sample needs to be carried out with outmost caution to avoid platelet activation. The assessment of PRP in the TGA-CAT method needs to be analysed close after blood sampling, as frozen platelets are lysed. In a study where f-PRP and ft-PRP was compared reached a conclusion stating there were no significant difference between the TGA-CAT parameters of the two (84). A finding that would make it possible to use ft-PRP in large multi-center studies. In our investigation we had a different statistical approach in that we used agreement analysis instead of correlation statistics by assessing the data with Bland-Altman plots (85). Our results showed a poor level of agreement between f-PRP and ft-PRP and we therefore cannot support the findings of Vila et al. (84). If their data would have been assessed with agreement analysis they may would have come to the same conclusion as we did. It is not uncommon that wrong type of statistical method is assessed in agreement analysis (85).

There are several TGTs available but few have been directly compared. We compared which of the two TGTs, TGA-CAT and INNOVANCE ETP, had the best correlation to FVIII:C in a cohort of PWH. The main difference between the TGTs is in the way they detect thrombin generation, TGA-CAT is a fluorogenic method while INNOVANCE ETP is a chromogenic method. Samples need to be defibrinated in the chromogenic method which is not needed in the fluorogenic method. Additionally, the platelet contribution to the global coagulation capacity can be assessed in the TGA-CAT but not in the INNOVANCE ETP method. Both methods showed an equal correlation to the FVIII:C activity. The correlation

between ETP of the two methods was poor. In a study by Sonnevi et al. (86) comparing the two TGTs reported a good correlation. However, a TF concentration of 10 pM was used in their study. In another study the best correlation between the two TGTs was achieved with 6 pM (CAT) and 300 pM (INNOVANCE). In the same study they found the INNOVANCE ETP method to exclusively detect extrinsic coagulation for all TF concentration measured (1-300 pM), while in the TGA-CAT intrinsic coagulation pathway amplification was measured at low concentrations (1 and 2.5 pM). In our comparison, we used 1 pM TF in the fluorogenic method and a low TF concentration in the chromogenic method. Not knowing the exact TF concentration of the INNOVANCE ETP method we cannot know if a difference in TF concentration led to the poor correlation between the two TGTs.

TGA-CAT results produced in Stockholm were compared with the Malmö results and assessed with agreement analysis yielding a poor level of agreement. The ETP variable showed the best agreement level with 29% and after normalization improved to 41%. Some of the factors that might have led to the poor level of agreement may be differences in analyzing temperature, pipette handling and resuspension of reagents.

The poor level of agreement for the TGA-CAT method found in paper II and equally poor results from unpublished inter-laboratory agreement analysis assessments inspired us to initiate our Nordic multi-center TGA-CAT standardization study. TGA-CAT Our standardization concept consisted of two phases: First, a pre-study on-site inspection, and second, the implementation. In the pre-study inspection, all participating laboratories were evaluated by the same study coordinator. During the visit, all equipment and materials were inspected, e.g. calibration of pipettes, software version and service protocols. Measurements of the room (RT) and instrument analyzing temperature were made. The local operator's and the inspector's TGA-CAT results were compared in a test run, that was performed to investigate the operator influence on the method variability. At the visits the laboratory protocol was further developed by implementing valuable feedback received from the operators.

In the implementation phase, all samples were run with two standardized reagents (PPP)-reagent LOW (TF 1 pM), and PPP-reagent (TF 5 pM). With the goal of covering the entire measuring range of the TGA-CAT method, three plasma types were assessed (*hypo*-, normal, and *hyper*- coagulable plasma). Five test runs were conducted with each PPP-reagent where each run consisted of three reference plasmas, four *hypo*- plasmas, four normal plasmas and four *hyper*- plasmas in triplicate.

We believe two things may have increased compliance to the laboratory protocol and thus contributing to our standardization concepts good results. First, the

operator involvement of the laboratory protocol development and second, the protocol being time point-based with a 50 minute countdown procedure. In a recent study by Hemker (87), he listed three substantial obstacles for the method to overcome of which the thermostability was the most significant one. In our pre-study, measurement of the analyzing temperature showed a 2.3°C difference between the center with the highest and the center with the lowest temperature. A 2.3°C difference in analyzing temperature is associated with 10-20% higher ETP and Peak values according to tests conducted at MCTH. Timed temperature measurements at MCTH have shown that it takes about 45 minutes for the Fluoroskan Ascent to reach its intended analyzing temperature of 37°C, a temperature that, according to the Thrombinoscope software, is reached within only 10 minutes. Based on these observations the operator is instructed to start the analyzer at least one hour before the start of incubation.

With the objective to investigate the effect of maximal physical exercise on specific and global coagulation parameters in healthy subjects and persons with severe HA, samples were taken before and after a maximal physical exercise test on a bicycle ergometer. We also investigated whether maximal physical exercise prolongs the effect of factor concentrate administered just before exercise. The results of the control group confirm the findings of previous studies, it is generally accepted that exercise in various forms leads to an increase in both coagulation and fibrinolysis in HS (58-62). A higher intensity of exercise is required for the pro-coagulant system to increase in comparison to fibrinolysis (88). The duration of the pro-coagulant system increase is thought to be longer than for fibrinolysis (61). It is confirmed by our findings, where FVIII:C, VWF:Ag, VWF activity and APTT remained changed 4 hours after exercise while t-PA did not. However, the prolonged elevations of FVIII:C and VWF was not reflected in TG where no significant changes remained after 4 hours. Global assays such as TGA-CAT and ROTEM have rarely been assessed to evaluate the effect of physical exercise and the results are conflicting. Significant increases in TG parameters after exercise have been reported by some (55), but not by others (89). We found significant changes that may be attributed to the marked intensity exercise. Even though the exercise induced increase in the FVIII:C appears to be the main mechanism behind the increased coagulability, there have been results presented with changes in several coagulation proteins in HS of a conflicting nature (55, 58, 59).

An increase in FII, FVII and fibrinogen levels after exercise in persons with severe HA was reported by Koch *et al.* (66). However, the results of our study enhances the essential role of FVIII in human subjects, as the global function of the coagulation system increased in controls but not in patients, where the exercise-induced increase in FVIII:C was absent.

The effect of exercise on VWF and FVIII clearance was investigated in a previous study in which young adults with severe HA were given FVIII concentrate with and without exercise. Their VWF:Ag levels increased immediately and remained elevated up to 6-8 hours after exercise while no decreased FVIII clearance was seen (90). In our study, persons with severe HA showed an almost identical increase in VWF levels as HS. Since higher levels of VWF have been shown to correlate with increased half-life of FVIII (63), we speculated that the increase in VWF activity would influence the PK parameters of FVIII concentrate administered before exercise.

However, no such changes were seen and, in addition, no effect on FVIII clearance was observed. This finding is in line with the results from a study by Zourikian et al. investigating the influence of moderate exercise on FVIII PK in 12 young adults with severe HA, who also failed to demonstrate any significant changes in PK parameters (90). Our and Zourikian et al. results, a VWF increase without effect on FVIII clearance, may be attributed to the small number of study subjects i.e. the studies were not sufficiently powered.

The limitations in the study include the small sample size and imperfectly matched controls (patients obtained significantly lower median peak heart rate and lower median relative  $VO_2$  peak than controls in the exercise test). Still, we do not believe these differences had any major impact on our results. Another limitation was not having analyzed all coagulation factor parameters in the patient group, a decision taken to minimize potential harm caused by increased risk of blood loss temporally close to strenuous exercise

# Conclusions and future perspectives

Our main findings were that:

- The TGTs, TGA-CAT and INNOVANCE ETP, correlate in an equal manner to the FVIII:C in PWH. The correlation between the two TGTs was poor. When dividing the cohort into disease severity, both methods fail to discriminate between them. Agreement analysis for the TGA-CAT method showed a poor level of agreement (Paper I).
- Assessing f-PRP and ft-PRP TGA-CAT results with agreement analysis shows a poor level of agreement (Paper II).
- The assessed TGA-CAT standardization concept produced agreement analysis results as robust as standard coagulation assays used in routine laboratories. Most of the plasma samples showed a decreased imprecision after being normalized by HemosIL RP. The dedicated normalization plasma aimed for the hypercoagulable measurement range, Affinity RP, performed even better than HemosIL RP. However, the dedicated normalization plasma aimed for the hypocoagulable measurement range, Siemens RP, did not perform better than HemosIL RP in that range. Thus, when analyzing plasma samples from populations where hypercoagulability is known or suspected normalization with Affinity RP could be considered. In populations with low or unknown coagulability, normalization with HemosIL RP could be considered (Paper III).
- Maximal physical exercise induced an increased global coagulation capacity in healthy individuals, whereas no change was obtained in persons with severe HA. The presence of a FVIII-independent mechanism that would increase global coagulation could not be found, our findings rather emphasizes the importance of FVIII in mediating the increase in coagulation capacity observed after exercise. The hypothesis that exercise induced increased levels of VWF in persons with severe HA could lead to a prolonged effect of factor concentrate administered just before exercise could not be supported by our results (Paper IV).

The TGTs have many potential clinical applications but most of them struggle with standardization issues. We have in this thesis shown that reproducibility levels associated with standard assays in routine laboratories now can be obtained for the TGA-CAT method when assessing our standardization concept. The development of a whole blood version of the TGA-CAT takes time but would of course, with more *in vivo* like analysis conditions, make the method yet more coveted. Future studies need to assess the reproducibility of the results obtained in our standardization study. A feature that might lead to an improvement of the standardization concept would be an extensive control of the analyzing temperature. A time and resource demanding procedure that by manipulation of the software temperature control would unify the analyzing temperatures of the participating laboratories. With the thermostability issues of the method in mind, it is reasonable to assume it would further improve our standardization concept. Hemker H.C. and his co-workers are working on the possibility to upgrade the heating system of the Fluoroskan Ascent analyzer with a thermoblock to address the thermostability issue. Our study on physical exercise effects on global and specific coagulation parameters in PWHAs needs to be repeated in a bigger cohort and to add the analyzing of specific coagulation factors needed to be able to evaluate exercise-induced effects on the fibrinolytic system. With the many new drugs on the market and several underway aimed for the treatment of both bleeding and thrombotic disorders, it will be interesting to follow to what extent thrombin generation will be assessed in the future drug management.



# Sammanfattning/Summary in Swedish

I denna avhandling utvärderas olika trombin genererings tests(TGT) förmåga att analysera hemofiliprover, med ett särskilt fokus på *thrombin generation assay-calibrated automated thrombogram* (TGA-CAT). TGT anses kunna ge en mer rättvisande bild av en individs totala koagulationskapacitet än vad fibrinbildnings baserade rutinanalyser kan. Detta då TGT mäter trombin koncentrationen under hela blödningsförloppet, från blödningens start till dess att den avstannat, medan fibrinklott baserade rutin metoder har sin mätändpunkt när endast 3-5% av trombinet har bildats. Många studier har visat på TGA-CAT metodens användbarhet i många olika sammanhang bl.a. vid diagnos och behandling av blödningsjukdomar, upptäckande av trombossjukdomar och vid monitorering och karakterisering av olika antikoagulant läkemedel. Problemet med dessa intressanta forskningsfynd är att de utförts på single-center nivå, d.v.s. i en liten skala. De skulle behöva upprepas i stora kliniska multi-center studier för att kunna uppnå den validitet som krävs av en metod för att bli framgångsrik och få spridning. I arbete I, ville vi undersöka vilken av två TGT, TGA-CAT och INNOVANCE ETP, som bäst korrelerade med FVIII aktiviteten i en samling hemofili A prover. Dessutom ville vi undersöka reproducerbarheten hos TGA-CAT metoden mellan de båda center med överensstämmelse analys. Det visade sig att båda TGT metoderna korrelerade på ett likvärdigt sätt mot FVIII:C,  $r=0.734$  (CAT) och  $r=0.701$  (ETP).

Överensstämmelse nivån för TGA-CAT metoden var dålig mellan de båda centerna. I arbete II, undersökte vi graden av överensstämmelse för TGA-CAT resultat från färsk trombocytrik plasma och fryst-tinad trombocytrik plasma för att kunna se om vi kunde bekräfta resultaten från en annan studie där det hävdades att det inte förelåg någon statistiskt signifikant skillnad mellan TGA-CAT resultat från färsk trombocytrik plasma och fryst-tinad trombocytrik plasma. Överensstämmelse analys visade på en låg nivå av överensstämmelse mellan färsk trombocytrik plasma och fryst-tinad trombocytrik plasma. Målet med arbete III var att höja TGA-CAT metodens låga standardiseringsnivå genom att utvärdera ett detaljerat standardiserings protokoll och tre normal plasma förmåga att normalisera resultat. Denna nordiska multicenter studie genererade resultat lika robusta som kan förväntas från koagulationsanalyser som används i rutin laboratorier. I det sista arbetet(arbete IV) ville vi undersöka effekten av fysisk aktivitet på specifika och globala koagulations parameter I en grupp på 10 personer med svår hemofili A och tio matchade friska försökspersoner. Vi undersökte också hur vida höjda VWF

nivåer efter fysisk aktivitet hade förmågan att förlänga effekten av FVIII-koncentrat som administrerats före fysisk aktivitet. Vi fann att maximal fysisk aktivitet genererade en ökad global koagulationskapacitet hos friska försökspersoner, medan ingen effekt kunde ses bland personer med svår hemofili. Våra resultat stöder inte hypotesen om att det skulle finnas en FVIII oberoende mekanism som ökar global koagulation, snarare understryks vikten av FVIII vid ökning av global koagulation. Sammanfattningsvis har våra resultat tillsammans bidragit till att flytta fram TGA-CAT metodens position ett steg närmare ett möjliggörande av stora kliniska multicenter studier.

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# Appendix



Start the analyzer 1 h before beginning the with step 1. if you want to start the moment you arrive at your laboratory make sure you have left the analyzer on over night.

Gather all material on the checklist(below) before you start the test run. Make and print a plate set-up using the Thrombinoscope software.

Prime the analyzer(set volume to 15000) with 37°C distilled water(50 ml tube).

When you are ready to begin with step 1 start a timer set on a 50 min countdown.

Bolded timepoints are more important to follow that un-bolded ones.

#### Important comments to certain steps

**Step 6.** With reverse pipetting technique aspirate 20 µl reagent and position the tip at the bottom of the well without touching the wall. Lift the tip 1-2 mm from the bottom and press to the first stop, then lift the tip straight up out of the "bubble" without touching the wall.

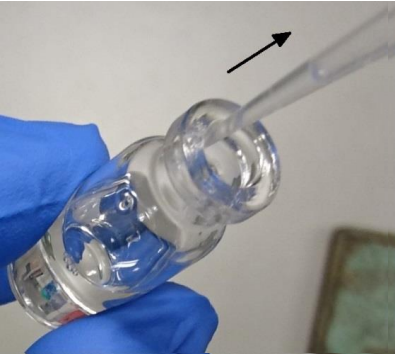
**Step 6-7:** When aspirating reagent/plasma try positioning the pipette tip as close to the surface as possible without aspirating air. When leaving the vial/tube allow the pipette tip to slide against the left side of the vial/tube edge to avoid getting drops of reagent/plasma formed on the outside of the pipette tip into the well (picture 1.).

**Step 7.** With reverse pipetting technique aspirate 80 µl plasma, the tip of the syringe should be lightly pressed against the well wall at the same time an equal pressure should be applied from the other side by a finger against the plate side (picture 2.).

**Step 11-12.** While working with the Substrate make sure to work swiftly and to try keep 37°C by holding the bottom of the tube firmly in your hand at all times (picture 3.).

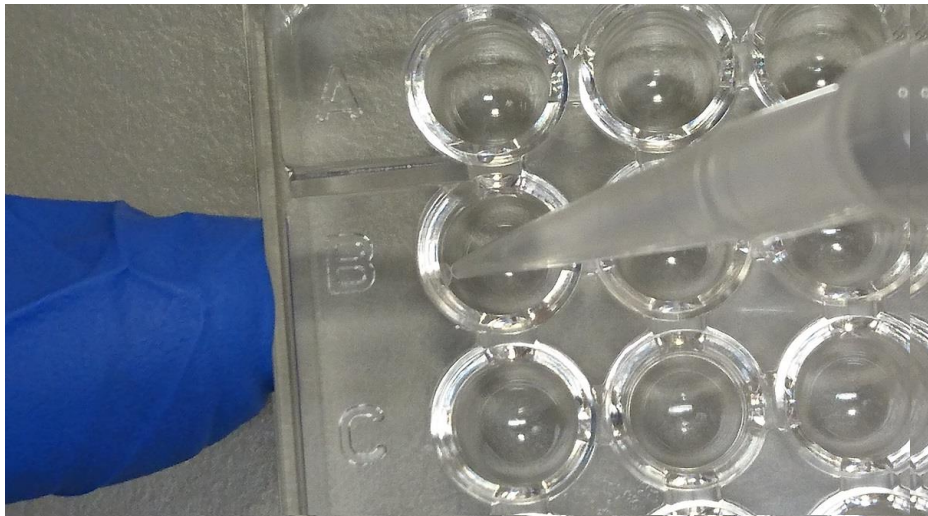
Time	Step	
<b>50</b>	<b>1</b>	<ul style="list-style-type: none"> <li>Room temperature (RT) reagents(Thrombin Calibrator, PPP-Reagent or PPP-Reagent LOW) and reference plasmas (HemosIL Calibration plasma, PS-LDP Affinity and Siemens Control Plasma P) and now start the 50 min countdown.</li> <li>Pipette 2600 µl Fluo-Buffer into a 14 ml U-bottom plastic tube, then cap it and place in a 37°C water bath.</li> </ul>
48	2	<ul style="list-style-type: none"> <li>Pour 10 ml distilled water into a screw cap flask for reconstitution of reference plasma and reagents.</li> <li>Fill 2 tubes(50 ml) with distilled water and place them in a 37°C water bath.</li> </ul>
<b>40</b>	<b>3</b>	<ul style="list-style-type: none"> <li>Reconstitute Siemens Control Plasma P by adding 5ml distilled water. • Reconstitute all other reagent and reference plasma vials by adding 1ml distilled water.</li> </ul>
35	4	<ul style="list-style-type: none"> <li>Invert all reconstituted vials and roll them carefully between your hands to make sure the content is fully reconstituted.</li> </ul>
<b>33</b>	<b>5</b>	<ul style="list-style-type: none"> <li>Thaw Hypo-, Normal- and Hyperplasma(Day 1-5) or Advate 1-5 and Adynovate 1-5 (Day 6-10) in 37°C water bath.</li> </ul>
<b>30</b>	<b>6</b>	<ul style="list-style-type: none"> <li>Take a plate and fill wells with reagents according to the provided plate set-up scheme using reversed pipetting technique (20µl). • Check that all wells are filled.</li> </ul>
<b>23</b>	<b>7</b>	<ul style="list-style-type: none"> <li>End thawing and vortex the plasma tubes. Fill the wells with plasma/reference plasma according to the provided plate set-up scheme by using reversed pipetting technique.</li> <li>Check that all wells are filled before moving on.</li> </ul>
<b>15</b>	<b>8</b>	<ul style="list-style-type: none"> <li>Put the plate in the analyzer and start the 10 min incubation.</li> </ul>
6	9	<ul style="list-style-type: none"> <li>Prepare the pipette that will be used for the substrate preparation by setting 65 µl.</li> </ul>
5	10	<ul style="list-style-type: none"> <li>Rinse the analyzer with 37°C distilled water (at least 5ml)</li> </ul>
4	11	<ul style="list-style-type: none"> <li>Add 65 µl Fluo-Substrate to the tube with 2600 µl Fluo-Buffer (37°C) and immediately vortex the solution thoroughly.</li> </ul>
<b>3</b>	<b>12</b>	<ul style="list-style-type: none"> <li>Now follow the on screen instructions without delay to keep the dispensing system and the substrate as warm as possible (picture 3).</li> </ul>
<b>1</b>	<b>13</b>	<ul style="list-style-type: none"> <li>When the syringe have been filled with substrate use a Kleenex to wipe it dry and check that the tip is straight before placing it in the hole marked "M" and press start.</li> </ul>
0	14	<ul style="list-style-type: none"> <li>When dispensing is done place the syringe in a 14 ml U-bottom plastic tube with enough distilled water to cover the tip of the syringe. • Put the pin back to the hole marked "M". • Place the dispenser tube in a 50 ml plastic tube with distilled water.</li> </ul>

Picture 1.



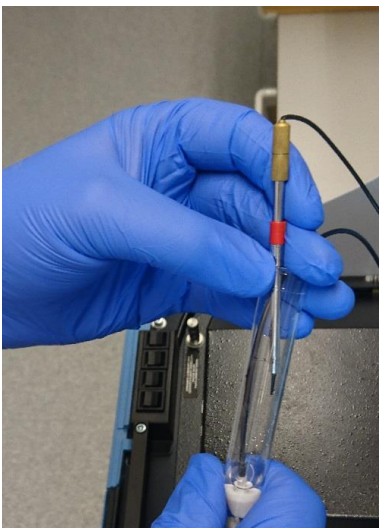
**Picture 1.** When leaving the vial/tube allow the pipette tip to slide against the left side of the vial/tube edge to avoid getting drops of plasma/reagent formed on the outside of the pipette tip into the well.

Picture 2.



**Picture 2.** Illustrates step 7. With reverse pipetting technique aspirate 80  $\mu$ l plasma, the tip of the syringe should be lightly pressed against the well wall (2-3 mm down from the edge) at the same time an equal pressure should be applied from the other side by a finger against the plate side.

Picture 3.



**Picture 3.** Illustrates step 12 by showing the handgrip to use when substrate is filled in the dispensing system. The tip of the syringe should be lightly pressed against the wall of the tube, otherwise foam will appear. The fingers of the other hand should embrace the lower part of the tube to keep the temperature of the substrate as close to 37°C as possible, still making visual control of the dispenser tube possible avoiding air bubbles in the dispensing system and suction to the bottom of the tube.













**Original Article**

**Correlation to FVIII:C in Two Thrombin Generation Tests: TGA-CAT and INNOVANCE ETP**

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**Competing interests:** The authors have declared that no competing interests exist.

**Abstract. Introduction:** Several thrombin-generation tests are available, but few have been directly compared. Our primary aim was to investigate the correlation of two thrombin generation tests, thrombin generation assay-calibrated automated thrombogram (TGA-CAT) and INNOVANCE ETP, to factor VIII levels (FVIII:C) in a group of patients with hemophilia A. The secondary aim was to investigate inter-laboratory variation for the TGA-CAT method.

**Methods:** Blood samples were taken from 45 patients with mild, moderate and severe hemophilia A. The TGA-CAT method was performed at both centers while the INNOVANCE ETP was only performed at the Stockholm center. Correlation between parameters was evaluated using Spearman's rank correlation test. For determination of the TGA-CAT inter-laboratory variability, Bland-Altman plots were used.

**Results:** The correlation for the INNOVANCE ETP and TGA-CAT methods with FVIII:C in persons with hemophilia (PWH) was  $r=0.701$  and  $r=0.734$  respectively. The correlation between the two methods was  $r=0.546$ .

When dividing the study material into disease severity groups (mild, moderate and severe) based on FVIII levels, both methods fail to discriminate between them.

The variability of the TGA-CAT results performed at the two centers was reduced after normalization; before normalization, 29% of values showed less than  $\pm 10\%$  difference while after normalization the number increased to 41%.

**Conclusions:** Both methods correlate in an equal manner to FVIII:C in PWH but show a poor correlation with each other. The level of agreement for the TGA-CAT method was poor though slightly improved after normalization of data. Further improvement of standardization of these methods is warranted.

**Keywords:** Thrombin generation test, Factor VIII, Hemophilia.

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**Introduction.** Routine laboratory analyses used to diagnose and monitor drug administration in bleeding disorders are primarily based on one-stage clotting assays and more recently, chromogenic assays. The one-stage clotting assay (based on clot forming endpoint) ends when only 3% of the total amount of thrombin of the coagulation process has been generated.<sup>1</sup> Thrombin is a key enzyme in the coagulation cascade, having both pro- and anti-coagulant abilities, so thrombin generation tests (TGTs) are considered to provide a more comprehensive picture of the patient's coagulation capability. The method has demonstrated utility in evaluating the overall hemostatic capacity both in bleeding and thrombotic disorders. There is at present reliable evidence that the method highly reflects the bleeding<sup>2-4</sup> or thrombotic<sup>5-10</sup> risk of patients with different coagulation disorders. It can also be used to predict the response to bypassing hemostatic agents administered to patients with hemophilia and inhibitors.<sup>11,12</sup>

There are several thrombin generation tests on the market, and most of them have not been compared to each other. The first aim of this study was to investigate which of two tests: the thrombin generation assay-calibrated automated thrombogram (TGA-CAT) or the INNOVANCE ETP, correlated best with the factor VIII level in a group of persons with hemophilia (PWH). TGA-CAT uses a fluorogenic substrate while the INNOVANCE ETP uses a chromophore for detection of thrombin generation. For the latter test, the plasma samples are defibrinated by its ETP reagent containing a fibrin aggregation inhibitor.<sup>13</sup> With the TGA-CAT method, this is not required.

Despite the advantages of TGTs, the TGA-CAT is used primarily in research laboratories due to the lack of standardization of the method and the large inter-center variability. However, in recent studies, a number of problems have been addressed, and promising improvements have been made in increasing the level of standardization for pre-analytical and analytical techniques.<sup>14-16</sup> Several investigations have also evaluated different standard reference plasmas' ability to reduce TGA-CAT inter-center variability.<sup>14,15,17</sup> The second aim of this study was to investigate the

inter-laboratory variability of the TGA-CAT method between two centers.

## Material and Methods

**Research subjects.** The study material was collected at two hemophilia care centers: The Hematology Center, Karolinska University Hospital, Stockholm, Sweden and the Hemophilia Centre, Belgrade, Serbia. Subjects had severe (<0.01 IU/ml), moderate (0.01-0.05 IU/ml) and mild (>0.05-<0.40 IU/ml) FVIII:C deficiency.<sup>18</sup> The samples from Stockholm were taken from 23 subjects (10 with mild, five with moderate and eight with severe hemophilia A). Patients with severe hemophilia from the Stockholm center were on prophylactic treatment. Time from the last dose of clotting factor was not standardized as samples were taken as part of routine visits. The samples from Belgrade were taken from 17 patients with hemophilia A (six with mild, five with moderate and six with severe disease) all of whom were on on-demand treatment.

Written informed consent was obtained from all subjects prior to the study. The study was approved by the Ethics Committee, Stockholm (Dnr 01-0003; 2006/778-32; 2013/263-32).

**Blood samples and plasma preparation.** Peripheral venous blood was collected into BD Vacutainer® plastic tubes (Becton Dickinson, Franklin Lakes, NJ, USA) with anticoagulant trisodium citrate (0.129 M, pH 7.4) (one part trisodium citrate and nine parts blood). Plasma was prepared within 60 minutes from venipuncture by centrifugation at 2000xG for 20 minutes at room temperature (RT), then divided into aliquots and stored at -70° C. Frozen samples on dry ice were transported from Belgrade to Stockholm and then from Stockholm to Malmö, and were still frozen and in good condition upon arrival.

**Methods.** TGA-CAT was measured according to the method described by Hemker et al.<sup>19,20</sup> Briefly, twenty microliters of PPP-reagent LOW (1 pM Tissue Factor (TF) and 4 µM phospholipids (PI)(TS31.00) and twenty microliters of Thrombin Calibrator (TS20.00) were manually pipetted into the wells of a round-bottom 96 well-microtiter plate (Immulon 2HB, Thermo Scientific, Rochester, NY, USA). All three reagents were

manufactured by Thrombinoscope BV, Maastricht, The Netherlands. Eighty microliters of plasma were added to each PPP-reagent well and its corresponding Thrombin Calibrator well.

The plate was then placed in a Fluorocan Ascent reader (Thermo Labsystems, Shanghai, China) for a 10 minute, 37°C incubation. Following the incubation twenty microliters of the starting reagent, FluCa-kit (TS50.00), was automatically dispensed into each well by the fluorometer. The wavelengths of 390 nm (excitation) and 460 nm (emission) were used to detect the fluorescence intensity. Thrombin generation curves were calculated by a dedicated software program, Thrombinoscope (Thrombinoscope BV, Maastricht, The Netherlands) version: V5.0.0.742. The TGA-CAT setup, instrumentation, software version, method and reagents, were identical for both laboratories. The intra-assay and inter-assay coefficients of variation (CV) for endogenous thrombin potential (ETP) with the TGA-CAT, Malmö, are 2.3% (n=12) and 9.5% (n=5), respectively.

The second thrombin generation test, INNOVANCE ETP, a chromogenic ETP assay (Siemens Healthcare Diagnostics, Marburg, Germany), was performed in Stockholm on a BCS XP system using C-settings according to the manufacturer's instructions and as previously described.<sup>21</sup> Dilutions of Innovin, 1:555 and actin, 1:20 are mixed in the proportion 1:2 and this mixture is used as an activator. The intra-assay and inter-assay CV for the area under the curve (AUC) with the INNOVANCE ETP method (C-setting) are 4,7% (n=5) and 5,7%(n=4) respectively.

The FVIII:C was determined with a FVIII assay, Coamatic (Chromogenix, Instrumentation Laboratory SpA, Milano, Italy) on a BCS XP Instrument (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) in Stockholm. In the test of agreement, the TGA-CAT parameters were normalized<sup>17</sup> by dividing the value obtained when analyzing each patient's plasma with that obtained when analyzing CryoCheck pooled normal plasma (Precision BioLogic, Dartmouth, Canada).

*Statistical analysis.* The associations between parameters were evaluated using Spearman's (non-parametric) rank correlation test. For the statistical presentation and evaluation of the TGA-CAT inter-laboratory variability, Bland-Altman

plots, 45-degree lines, and frequency plots were used.

## Results

*Correlations.* The correlation between the TGA-CAT parameter ETP and the corresponding parameter for INNOVANCE ETP AUC was  $r=0.546$ .

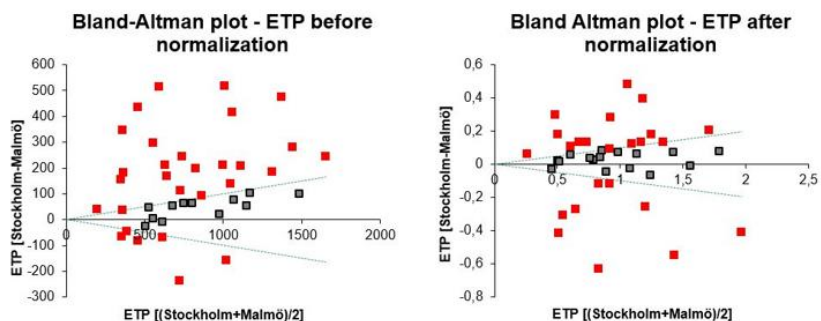
Both methods (ETP and AUC) showed a similar correlation to FVIII:C ( $r=0.734$  and  $r=0.701$ ). The FVIII:C values were divided according to disease severity (severe, moderate and mild) and the results for FVIII:C to ETP and AUC at the group level are shown in **Table 1**. Results grouped by severity showed lower associations compared to the total sample.

**Table 1.** Correlation coefficient (r) for ETP and AUC to FVIII:C in all patients and grouped by disease severity.

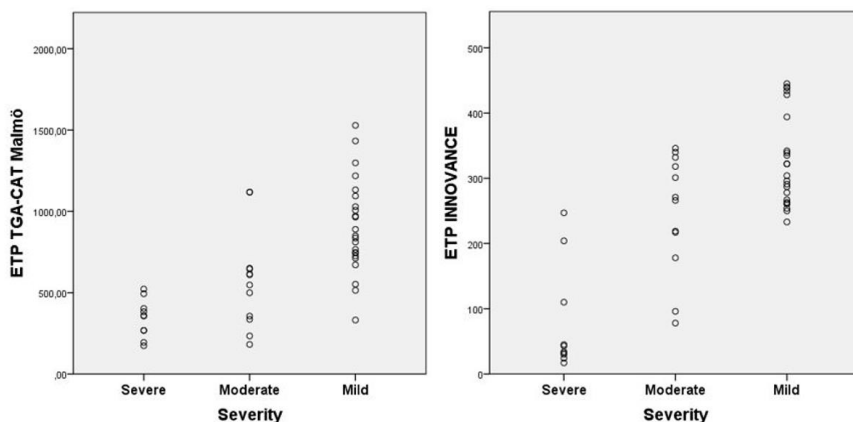
	FVIII:C			
	Severe	Moderate	Mild	All patients
<b>TGA-CAT ETP</b>	0.240	0.172	0.458	0.734
<b>INNOVANCE ETP AUC</b>	0.185	0.305	0.238	0.701

To determine the precision in terms of severity, FVIII levels were grouped as severe, moderate and mild (laboratory severity) and plotted against ETP values (**Figure 1**). Substantial overlap in the groups was observed with both assays, indicating that neither of the methods was considerably better in discriminating among categories of disease severity.

*Test of Agreement: TGA-CAT Malmö vs. Stockholm.* When performing inter-laboratory tests of agreement, a difference of  $\pm 10\%$  is considered to be an acceptable level by most laboratories. In most cases, 95% of the observations need to be within the  $\pm 10\%$  acceptance level.<sup>22</sup> The variability between the TGA-CAT results performed in Malmö and Stockholm was rather extensive, but after normalization it was reduced. The results for the ETP parameter are presented in Bland-Altman plots, before and after normalization (**Figure 2**). Before normalization, 29% of the samples were within the  $\pm 10\%$  cone of acceptance. Normalization of the data improved results to 41%, still far from the 95% acceptance level. The results for peak, lag time (LT) and time to peak (tpeak) are inferior to that of the ETP parameter (**Table 2**).



**Figure 1.** Patients grouped by severity of hemophilia A and plotted against ETP for both methods, TGA-CAT Malmö and ETP INNOVANCE (AUC).



**Figure 2.** Before normalization: Observations within the green dashed cone represent a  $\pm 10\%$  or less difference between ETP values of Stockholm and Malmö results. 29% of the observations lie in the  $\pm 10\%$  cone of acceptance. After normalization: Observations within the green dashed cone represent  $\pm 10\%$  or less difference between normalized ETP values of Stockholm and Malmö results. 41% of the observations lie in the  $\pm 10\%$  cone of acceptance.

**Table 2.** Level of agreement between Malmö and Stockholm TGA-CAT parameters, before and after normalization.

	ETP	peak	lagtime	ttpeak
Before normalization	29%	15%	0%	20%
After normalization	41%	32%	30%	39%

**Discussion.** In this study, two thrombin generation methods (TGA-CAT and INNOVANCE ETP) were compared. In our comparison, we choose to focus on ETP and AUC (ETP). Our evaluation showed poor correlation for ETP between the two TGTs. Another study comparing the two reported a good correlation,<sup>23</sup> however, this result was obtained with a higher TF concentration, 10 pM.

By inhibiting the intrinsic coagulation with an anti-factor VIII antibody, Devreese et al. showed that the INNOVANCE ETP detected extrinsic coagulation exclusively for all TF concentrations tested (1-300 pM), while in TGA-CAT the amplification of the intrinsic pathway was measured at low TF concentrations (1 and 2.5 pM).<sup>13</sup> In our comparison, we used 1 pM TF in the fluorogenic method (CAT) and a low TF concentration in the chromogenic method (INNOVANCE).

Since we do not know the exact TF concentration for the INNOVANCE ETP method, we cannot know if the difference in TF concentration had a part in the lack of correlation between the methods.

To achieve the desired number of samples for study, specimens from PWH in both Belgrade and Stockholm were used. All plasma samples were single centrifuged, thus, the chance of small amounts of platelets remaining in the plasma cannot be excluded. According to a study by Loeffen, et al.<sup>16</sup> TGA-CAT results were only affected by double centrifugation when the TF concentration was 1 pM or lower. Since our TF concentrations for the TGA-CAT method were 1 pM and 0.5 pM we cannot rule out the possibility that results may have been affected by the single centrifugation.

We did not, however, see a correlation between platelet counts and ETP values (results not shown) which indicates that single centrifugation, instead of the recommended double, did not have a significant impact on the results obtained. There are numerous reports describing contact activation as a reason for the poor reproducibility of TGA-CAT results<sup>16,24,25</sup> and it has been proposed that CTI (corn trypsin inhibitor) should be used for blood sampling. CTI was not used in this study, given the report by Spronk, et al.<sup>26</sup> stating that the addition of CTI, preventing the contact activation pathway, can only be motivated when TF concentrations are 0.5 pM or lower.

Further, we investigated the level of agreement when the same TGA method (TGA-CAT) was performed at two centers (Stockholm and Malmö). The inter-laboratory variation was decreased for all four parameters after normalization with pooled normal plasma, where the ETP results showed the highest concordance, 29% without normalization and 41% with normalization (**Table 2**). Even after normalization more than half of the samples did not reach the level of acceptance. The choice of centrifugation method, blood sampling tubes, and some other pre-analytical factors are of no concern when conducting agreement studies. Of crucial importance is that the characteristics of the plasma are identical for all samples at the start of the analysis. That said, some pre-analytical factors are influential, such as transportation, thawing, resuspension of reagents, pipetting, and time scheme from the end of thawing to start of analysis. In our investigation, thawing was performed identically, 37°C for 10 minutes. However, we did not have full control of the other factors that may have contributed to the low level of agreement. Interestingly enough, the factor that may have had the greatest impact on the results is

one that is out of the control of the lab technician, that is, the analyzing temperature of the measuring equipment. In a report by De Smedt, et al.<sup>27</sup> the importance of pre-heating was shown, leading to a ten-minute 37°C incubation step before the start of measurement in the latest software version for the method (version: V5.0.0.742). A post-study service the Fluoroscan Ascent reader in Malmö showed a temperature deviation of almost three degrees below the intended and displayed 37°C. Identical measurements were performed at two other Swedish laboratories by the same service engineer using the same measuring equipment. Measurements were approximately 1°C above and 1°C below ours. No temperature data from Stockholm was available, but deviation from the intended assay temperature is one possible reason for our large inter-laboratory variability. These divergent measurements indicate the need for temperature calibration in laboratories participating in multicenter studies.

The choice of using 95% of the observations within  $\pm 10\%$  as a quality standard for the whole measurement range of the TGA-CAT method could be argued. In several routine coagulation assays, a wider acceptance range is used for measurements in the outskirts of the methods measuring capacity, with acceptance ranges of up to  $\pm 15\text{-}20\%$  in its high and/or low measurement ranges. It might be justified to use a similar approach for the TGA-CAT method.

The main study limitation is the relatively small number of samples. That might explain the poor discrimination between the disease severity groups.

To conclude, both methods correlate in an equal manner to FVIII:C in PWH but show a poor correlation with each other. When dividing the study material into disease severity groups, both methods fail to discriminate between them. The inter-center variability for TGA-CAT method showed a low level of agreement.

Earlier studies have shown that through enhanced standardization of the assay and pre-analytical factors, the inter-laboratory variability can be reduced to acceptable levels and therefore open up the possibility of conducting multi-center clinical studies.<sup>13,14</sup> Still, further improvement of standardization is warranted for this method.

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# Paper II





ORIGINAL ARTICLE *Laboratory science*

# Low agreement between fresh and frozen-thawed platelet-rich plasma in the calibrated automated thrombogram assay

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**Introduction:** Thrombin generation tests (TGTs) are considered to give more detailed information of the overall coagulation capability of a patient than clotting-based routine assays. The TGT thrombin generation assay-calibrated automated thrombogram (TGA-CAT) uses both platelet-poor plasma (PPP) and platelet-rich plasma (PRP). Assessing PRP gives more physiological test conditions and is of great interest considering the important role platelets play in haemostasis. However, PRP needs to be assessed close after blood draw/preparation as freezing fragments the platelets. In several previous publications, the utility of frozen-thawed PRP (ft-PRP) has been promoted, and in one article, no significant difference between fresh PRP (f-PRP) and ft-PRP was reported. **Aim:** The aim of our study was to investigate the level of agreement between f-PRP and ft-PRP to further validate these results. **Methods:** Our test population contained 41 persons with haemophilia and 45 healthy subjects. We used the TGA-CAT method with a set-up according to the manufacturer of the method. **Results:** The measurements showed a poor level of agreement between f-PRP and ft-PRP and differences were not systematic. **Conclusion:** Fresh and ft-PRP cannot be assumed to show equal results in the TGA-CAT assay.

**Keywords:** frozen-thawed platelet-rich plasma, haemophilia, test of agreement, thrombin generation assay-calibrated automated thrombogram, thrombin generation

## Introduction

Haemophilia is an X-linked genetic disorder resulting from the deficiency or absence of functional coagulation factor VIII or factor IX, necessary for efficient blood clotting. Most routine coagulation assessments are based on one-stage clotting assays, for example prothrombin time (PT) and activated partial thromboplastin time (APTT) which both use the formation of a fibrin clot as the endpoint of the test. Global coagulation tests, such as the thrombin generation assay-calibrated automated thrombogram (TGA-CAT), monitor the concentration of thrombin throughout the entire coagulation process. They have therefore been proposed to be better tools for the detection of hypo- and hypercoagulability and for monitoring a patient's

drug administration compared to fibrin clot formation which only measures 3% of the total thrombin generated during the coagulation process [1].

In a longitudinal study, it has been shown that approximately 10% of patients with severe haemophilia exhibit a mild phenotype with no spontaneous joint bleeding events [2]. Moreover, patients with mild haemophilia may, in some cases, exhibit a severe phenotype despite having factor levels >5% [3]. By comparing TGA values in families with discordant vs. concordant phenotypes, it has been suggested that TGA predicts the clinical phenotype in haemophilia better than factor level alone [4].

TGA is widely used in research laboratories, but due to a lack of standardization of the method and the large interlaboratory variability, TGA-CAT has not commonly been used in multicentre clinical studies. In some later studies, however, problems have been addressed and promising improvements have been made [5,6].

Both platelet-poor plasma (PPP) and platelet-rich plasma (PRP) are used when running TGA-CAT assays. Given the important role of platelets in blood

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coagulation, PRP will likely give a more detailed picture of the patient's coagulation capability than PPP.

An inconvenience associated with use of PRP is that it must be analysed soon after blood sampling, as frozen cells are lysed. The use of frozen-thawed PRP (ft-PRP) has been questioned in several articles [7,8]. However, in a study in which fresh PRP (f-PRP) was compared to ft-PRP there were no significant differences between the parameters, opening up opportunities for the use of ft-PRP in multicentre studies [9]. The aim of our study was to investigate the level of agreement between f-PRP and ft-PRP to further validate these results.

## Materials and methods

### Subjects

Blood samples were collected from 41 persons with haemophilia (PWH). Thirty-three had severe haemophilia A (factor VIII level  $<0.01$  kIU L<sup>-1</sup>), six had severe haemophilia B (factor IX level  $<0.01$  kIU L<sup>-1</sup>), one had moderate haemophilia A (factor VIII level 0.01–0.05 kIU L<sup>-1</sup>), and one had mild haemophilia B (factor IX level  $>0.05$  to  $>40$  kIU L<sup>-1</sup>). Blood samples were also collected from 45 healthy control subjects (HS). Written informed consent was obtained from all subjects and controls prior to enrolment into the study. The study was approved by the Regional Ethics Committee of Lund University, Sweden (Dnr 2012/373).

### Blood sampling

Blood samples were obtained by venipuncture and collected into BD Vacutainer<sup>®</sup> plastic tubes (Becton Dickinson, Plymouth, UK) with anticoagulant sodium citrate, 105 mM. To obtain PRP, blood samples were centrifuged at  $200 \times g$  for 10 min at room temperature (RT). Platelet count was adjusted at  $300 \times 10^3 \mu\text{L}^{-1}$  by addition of PPP in the first 20 participants. To harmonize with recommendations from the manufacturer, platelet count was adjusted to  $150 \times 10^3 \mu\text{L}^{-1}$  in 66 subjects. To verify that the platelet count did not have a major impact, TGA parameters were measured in PPP spiked with different concentrations of normal platelets. To obtain PPP, blood samples were first centrifuged at  $2000 \times g$  at RT for 20 min. The supernatant was then re-centrifuged for an additional 20 min at  $2000 \times g$ .

A batch of PRP and PPP was processed within 2 h after venipuncture, and remaining samples were stored at  $-80^\circ\text{C}$  to permit subsequent analysis of samples.

### Thrombin generation

Thrombin generation was measured according to the method described by Hemker *et al.* [10]: calibrated

automated thrombogram (CAT; Thrombinoscope BV, Maastricht, the Netherlands).

In summary, 20  $\mu\text{L}$  of PPP-Reagent LOW [1  $\mu\text{M}$  tissue factor (TF)], PRP-Reagent (0.5  $\mu\text{M}$  TF) and Thrombin Calibrator (TS31.00, TS42.00 and TS20.00; Thrombinoscope BV) was dispensed into the wells of a round-bottom 96-well microtiter plate (Immulon 2HB; Thermo Scientific, Rochester, NY, USA). Eighty microlitres of plasma, fresh or frozen-thawed, was added, and then, the plate was placed in the Fluorometer for a 10 min,  $37^\circ\text{C}$  incubation (Fluoroskan Ascent; Thermo Scientific, Shanghai, China, equipped with a 390/460 filter set). Prior to the start of the analysis, 20  $\mu\text{L}$  of fluorogenic substrate with calcium (TS50.00 FluCa-kit; Thrombinoscope BV) was added by the analyser. Thrombin generated was quantified using the READER software (Thrombinoscope; Thrombinoscope BV) version: V5.0.0.742. All samples were analysed in triplicate. All frozen plasma was thawed for 10 min in a  $37^\circ\text{C}$  water bath.

### Statistical analysis

When conducting interlaboratory tests of agreement, a difference of  $\pm 10\%$  is often considered to be an acceptable level. In most cases, 95% of the observations need to be within the  $\pm 10\%$  acceptance level to be valid for routine use in laboratories [11].

Statistical analyses were performed for Peak and ETP (Endogenous thrombin potential). To study the agreement of f-PRP and ft-PRP, a Bland–Altman plot was used showing the agreement between fresh and frozen-thawed plasma. For descriptive statistics, the percentages were presented for measurements inside the  $\pm 10\%$  area of acceptance.

## Results

The following parameters are calculated when using the READER software: lag-time, time to peak, Peak, ETP, start-tail, VelIndex, tReliability and Alpha2M\_Level. Of the four parameters considered of greatest clinical importance (lag-time, peak, time to peak and ETP) [12], we focus on ETP and Peak in the presentation of our results.

### TGA parameters in PRP at different platelet concentrations

To ensure that the platelet level in PRP did not have a major impact on TGA parameters, TGA analysis was performed on PPP spiked with different concentrations of platelets. As seen in Fig. 1, TGA parameters seemed to plateau at levels above  $150 \times 10^3 \mu\text{L}^{-1}$  which has been reported in earlier studies [13,14], allowing for the grouping of samples with different platelet counts into one cohort.

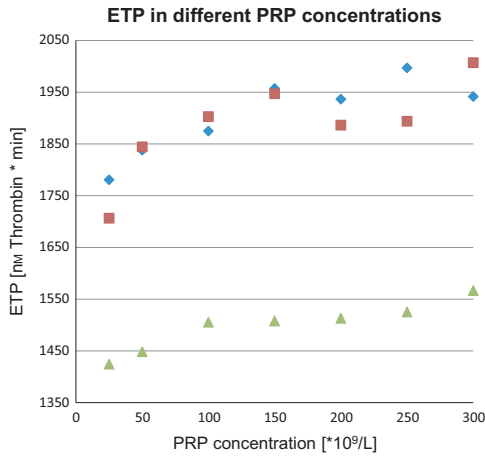


Fig. 1. PPP was spiked with different volumes of platelet concentrate to obtain the platelet levels indicated on the Y axis. ETP was then measured in all samples.

*Test of agreement*

In previous studies, TGA-CAT has been shown to have substantial interlaboratory variability. Difficulty in achieving  $\pm 10\%$  agreement for 95% of observations in comparisons from different laboratories is a clear indication of a need for further standardization [15,16].

The test of agreement results between fresh and frozen-thawed PRP and PPP in ETP and Peak in our study can be seen in Table 1, presented as percentage of observations within the  $\pm 10\%$  acceptance margin. None of the results from the groups examined reached the desired 95%. For the entire study population, Peak and ETP results for PRP were 7% and 47%, respectively. These are displayed by Bland–Altman plots in Fig. 2. Corresponding results for the PWH group were 15% and 29%.

ETP in PRP for the HS group showed the highest level of agreement: 62% of the observations lay within the acceptance margin. The corresponding result for PWH was 29% (Fig. 3).

Comparisons of f- and ft-PPP results in both ETP and Peak for all groups showed that only 18–34% of observations were within the 10% cone of acceptance.

**Discussion**

We investigated the level of agreement between f-PRP and ft-PRP. When examining agreement between parameters, plots that provide a visual display of the distribution of differences; for example Bland–Altman plots, plots against a 45-degree line, are commonly used. When data are approximately normally

Table 1. Percentage of observations for ETP and Peak in PPP and PRP that are within the  $\pm 10\%$  acceptance margin.

	All (%)	PWH (%)	HS (%)
PRP			
ETP	47	29	62
Peak	7	15	0
PPP			
ETP	26	34	18
Peak	20	22	18

PWH, persons with haemophilia; healthy control subjects; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

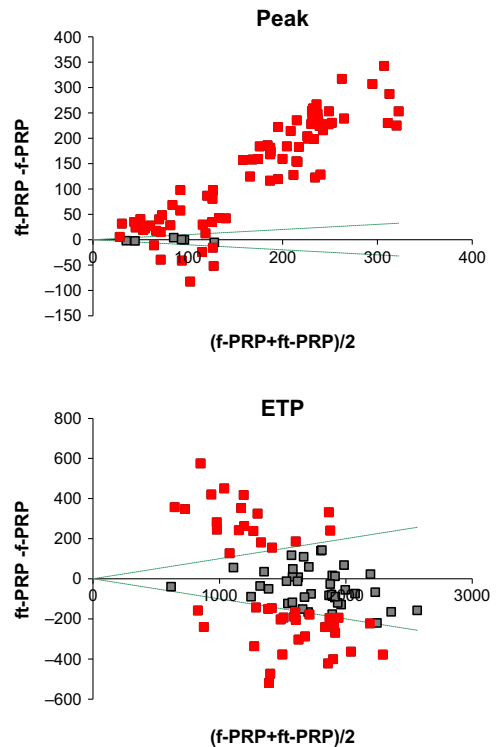


Fig. 2. Comparison of measurements of the TGA-CAT parameter Peak (upper panel) and ETP (lower panel) between ft-PRP and f-PRP. Observations within the green dashed cone represent  $\pm 10\%$  difference between ft-PRP and f-PRP. Seven and 47% of the observations lie within the 10% cone of acceptance for the Peak and ETP parameter, respectively.

distributed, a confidence interval (CI) could also be calculated [17]. The use of correlations when studying the level of agreement can be misleading because a correlation measures the strength of a relationship but not the agreement. If there is a systematic difference between the data points, the correlation coefficient will be high, but the level of agreement will still be low.

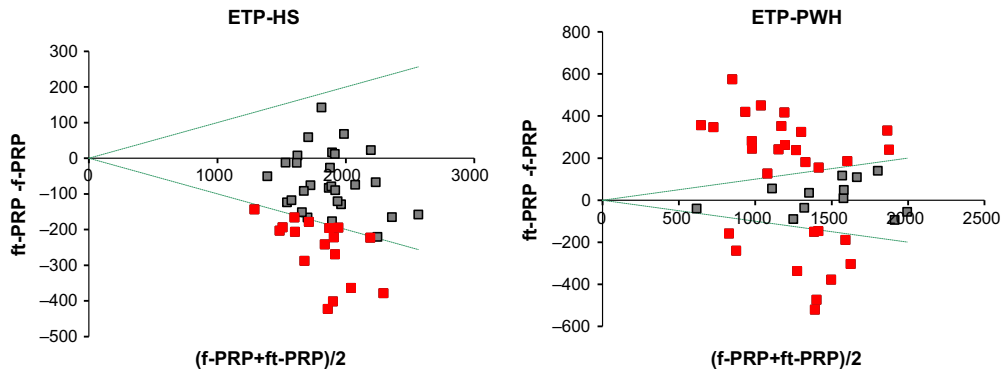


Fig. 3. Comparison of measurements of the TGA-CAT parameter ETP in PWH (upper panel) and HS (lower panel) between ft-PRP and f-PRP. Observations within the green dashed cone represent  $\pm 10\%$  difference between ft-PRP and f-PRP. Sixty-two and 29% of the observations for the ETP parameter lie in the  $\pm 10\%$  cone of acceptance in HS and PWH, respectively.

In our study, the highest level of agreement was found in ETP for PRP among the HS group (62%), while the corresponding result for the PWH group was only 29% (Fig. 3). The observations in the HS group outside the  $\pm 10\%$  cone were all gathered in the lower part of the cone (CV 91.6%), while PWH group observations were less consistent, with results spread in both the upper and lower part of the cone (CV 343.6%) (Fig. 3), meaning that TGA parameters in ft plasma may as well have a higher as a lower value as compared to fresh plasma. The low agreement in the PWH group indicates that the level of FVIII/FIX influences the variability of the method when fresh and ft-PRP are compared.

The level of agreement for ETP and Peak values between f-PRP and ft-PRP was low. The desired outcome of 95% of observations deviating less than  $\pm 10\%$  was not obtained for any of the groups or parameters tested [11]. In a similar study by Vila *et al.* [9], no significant differences between f-PRP and ft-PRP were found, but the level of agreement was not determined. As systematic differences between observations will result in a high degree of correlation and the absence of significant difference between groups may be attributable to a small number of observations, it cannot be ruled out that the level of agreement was also low in the Vila study.

Frozen-thawed platelets are disrupted into fragments and should not provide the same platelet attributes to the clotting system as fresh platelets. Thus, it is not surprising that global tests use either fresh whole blood or frozen platelet-poor plasma [18]. Regnault *et al.* [19] found a slight but significant difference between the TGA-CAT parameter ETP in f-PRP and ft-PRP.

For coagulation analyses, and for global coagulation analysis in particular, it is of great importance to be in control of pre-analytical parameters. One could

speculate as to whether different pre-analytical conditions, for example type of blood collection tubes, centrifugation and blood sampling methods, would have raised the level of agreement in the current study. Corn trypsin inhibitor (CTI) blood collection tubes inhibit FXIIa, thus blocking the contact activation pathway, and have been used by many centres to minimize the influence of pre-analytical factors. Spronk *et al.* [20], however, reported that addition of CTI is only useful when triggering TF concentrations of 0.5  $\mu\text{M}$  or lower. We used a standardized trigger for PPP, PPP-Reagent LOW [1  $\mu\text{M}$  TF, 4  $\mu\text{M}$  phospholipids (Pl)], recommended for plasmas from haemophiliacs as almost half of our test plasmas were taken from PWH. Our PRP trigger was also standardized, PRP-Reagent (0.5  $\mu\text{M}$  TF, 0  $\mu\text{M}$  Pl). In our study, the use of CTI was not considered necessary as the reagents TF concentration exceeded 0.5  $\mu\text{M}$ . However, whether minimizing the contact activation by CTI might result in a higher level of agreement remains to be tested in future studies. Interestingly, the level of agreement between f-PPP and ft-PPP was in the same low range as that using PRP. This could be explained by smaller amounts of residual platelets being present in our PPP. When using plasmas stored without precautions relating to platelet contamination, for example in epidemiological studies, Chantarangkul *et al.* [21] showed that using filtration after thawing and before testing the unwanted effect of residual platelets could be avoided.

## Conclusion

To conclude, our study showed a poor level of agreement between f-PRP and ft-PRP as well as between f-PPP and ft-PPP TGA results and did not support findings reported by Vila *et al.* [9]

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## Contributions

Marcus Ljungkvist designed the study, performed the experiments and wrote the manuscript. Susanna Lövdahl designed the study, performed

statistical calculations and wrote the manuscript. Eva Zetterberg designed the study and wrote the manuscript. Erik Berntorp designed the study and wrote the manuscript.

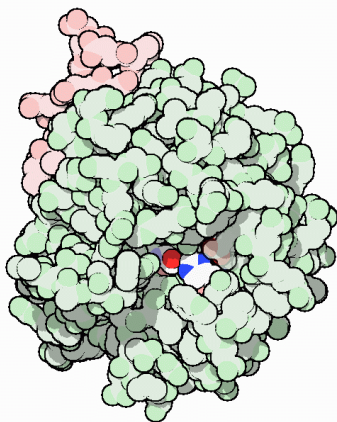
## Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Thrombin is a serine protease: a protein-cutting enzyme that uses a serine amino acid to perform the cleavage. The active site is seen here, at the base of a deep groove. The oxygen atom of the key serine amino acid is shown in bright red, and the two bright blue nitrogen atoms are part of a histidine that activates the serine. Thrombin is the key enzyme of the coagulation cascade acting both as a procoagulant and as an anticoagulant. As a procoagulant, thrombin promotes conversion of fibrinogen into an insoluble fibrin clot. In addition, it activates FV, FVIII, FXI and FXIII. As an anticoagulant, its interaction with thrombomodulin leads to the activation of protein C that in turn down-regulates thrombin generation by inactivating FVa and FVIIIa.

