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DISSERTATION

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Chapter 1

General introduction & Outline

HAEMOSTASIS

Haemostasis is an important physiological process that enables blood to form a clot when an injury occurs [1,2]. In pathophysiology, an imbalance in this process can lead to the formation of an unwanted blood clot in a non-injured blood vessel, known as thrombosis, or at the other side of the spectrum to the lack of blood clot formation at a wound, which leads to unwanted blood loss [3-5]. The haemostatic process is triggered by a damaged vessel wall, followed by adhesion, activation and aggregation of platelets to the exposed sub-endothelial extracellular matrix (Figure 1) [6-8]. An important part of the haemostatic system is provided by the coagulation cascade. Herein, coagulation factors are on of proteolytically active thrombin [9-11]. Furthermore, activated platelets expose phosphatidylserine on their outer membranes, providing a major surface for activated in a cascade like fashion, eventually leading to the formation of thrombin generation [12,13]. The interaction of activated platelets and coagulation factors forms a fibrin clot, which stops the blood leakage at a site of injury [14,15].

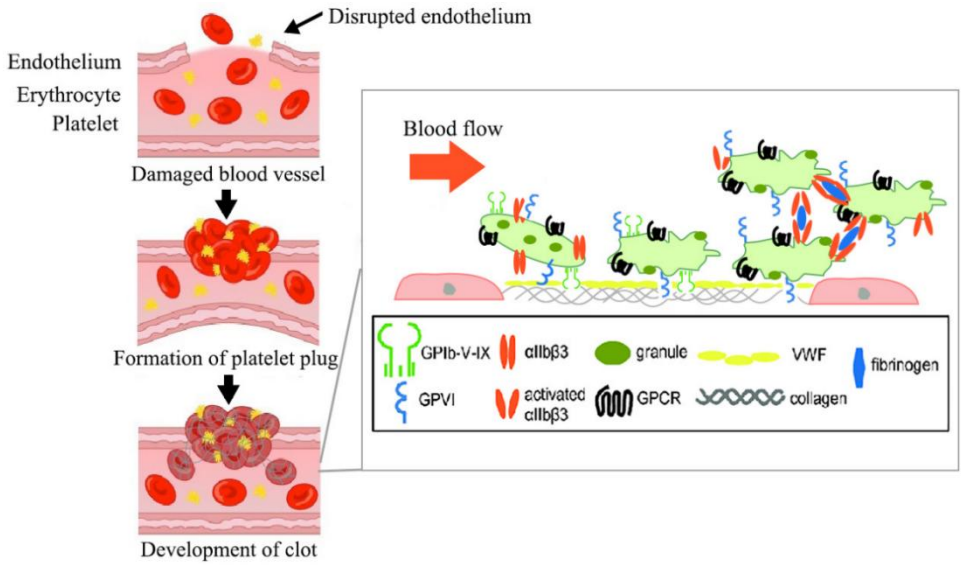

Figure 1. Overview of the primary haemostasis process. Once a blood vessel gets injured,

circulating platelets adhere to the site of injury. Through a variety of receptors - glycoprotein (GP)IbV-IX, GPVI and G-protein-coupled receptors (GPCR), the platelets become activated, which leads to platelet-platelet interaction via integrin αIIbβ3 and fibrinogen, to form a loose platelet plug. Activated platelets can also express procoagulant phosphatidylserine for the promotion of thrombin generation. Thrombin further activates platelets and coagulation factor, and in addition cleaves fibrinogen into fibrin, which results in the formation of a platelet-fibrin clot.

THE COAGULATION CASCADE

Thrombin is considered to be a central enzyme of the blood coagulation cascade. Thrombin activates platelets, several coagulation factors, and it furthermore cleaves fibrinogen into fibrin to form a fibrin clot [16-19]. The formation and inactivation of proteolytically active thrombin is strongly regulated to control the haemostatic process [20,21].

The generation of thrombin can be triggered via two pathways: the extrinsic and intrinsic coagulation pathway (Figure 2) [22]. The extrinsic pathway is triggered by exposure of tissue factor (TF) at the site of vessel wall injury. TF binds and activates factor (F) VII and thereby forms the extrinsic tenase complex. This complex activates FX into FXa [23,24]. The produced FXa combines with co-factor FVa to form the prothrombinase complex, which evokes the first traces of thrombin.These trace amounts of thrombin activate FXI into FXIa, and subsequently induce the generation of FIXa. Together with FVIIIa and phospholipids, FIXa forms the intrinsic tenase complex, which generates more FXa. The phospholipid-dependent formation of more prothrombinase complexes (FXa-FVa) results in a thrombin burst that massively converts fibrinogen to fibrin [2]. Thrombin can reinforce its own production through the activation of FV, FVIII, FXI.

As also shown in Figure 2, thrombin is inhibited by the natural anticoagulants, antithrombin and α_2Macroglobulin (α_2M) [25]. Antithrombin is a serine protease inhibitor, which potently inactivates thrombin and FXa, and to some extent also FIXa, FXIa and FXIIa [26,27]. α_2M is a plasma protein mainly produced by the liver that inactivates a variety of proteinases, such as thrombin. Antithrombin inhibits approximately two-thirds of all

thrombin formed, while α_2M inactivates one-quarter of the formed thrombin [28]. The remaining thrombin is inhibited by a group of miscellaneous serpin and non-serpin inhibitors [29].

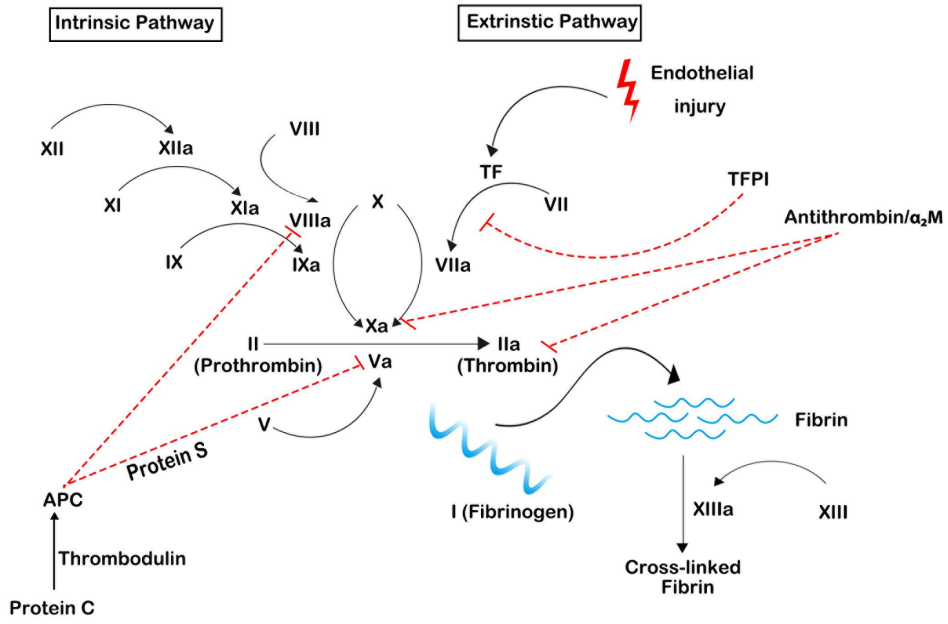

Figure 2. Simplified scheme of the coagulation. FVII is activated upon binding to tissue factor (TF) following endothelial injury. The TF-FVIIa complex converts FX to FXa. FXa converts a small amount of prothrombin to thrombin, which activates FV. FXa binds to FVa, leading to a burst of thrombin formation. The intrinsic pathway begins with the contact-dependent activation of FXII, which in activated form converts FXI into FXIa. FXIa activates FIX, which together with co-factor FVIIIa forms initial tenase complexes, thereby activating FX to FXa. In the presence of phospholipids, additional tenase complexes accelerate the conversion of prothrombin to thrombin, which yields fibrin from fibrinogen. The main anticoagulant pathway involves thrombomodulin-dependent activation of protein C into activated protein C (APC), which inhibits FVa and FVIIIa. The procoagulant and anticoagulant pathways are indicated in black and red, respectively. Antithrombin and $\alpha_2$macroglobulin (α_2M) inactivate the formed thrombin and FXa. The activation of FX by

TF-FVIIa is also inhibited by tissue factor pathway inhibitor (TFPI). APC inhibits FVa and FVIIIa by proteolytical cleavage.

The plasma level of α_2M is higher in children, while it also increases in pathological conditions, as in patients with liver cirrhosis. The α_2M-dependent thrombin inactivation can also increase under conditions where the antithrombin level is unchanged [30,31]. It is considered that the rise of α_2M level is a protective mechanism in case of antithrombin deficiency.

The protein C pathway is another important anticoagulant process. In this anticoagulant pathway, thrombin binds to thrombomodulin, which transforms the procoagulant function of thrombin into an anticoagulant one: the activation of protein C (APC) [32]. By its proteolytic activity APC is a potent inhibitor of FVa and FVIIIa, thereby inhibiting the formation of additional thrombin in two ways: directly by inhibiting the prothrombinase complexes (FXa-FVa) and indirectly through inhibition of the intrinsic tenase complexes (FIXa-FVIIIa) [2,33]. Another natural anticoagulant is provided by tissue factor pathway inhibitor (TFPI). TFPI binds to FXa and TF-FVIIa simultaneously and thereby prevents new formation of FXa [34,35].

PLATELET AND COAGULATION

Platelets play a major role in localizing and controlling the burst of TG and subsequently clot formation [14,36]. In addition to the damaged vessel wall, platelets provide the major surface for TG by exposing phosphatidylserine on their outer membrane, on which the procoagulant enzyme complexes assembly when activated, i.e. the tenase and prothrombinase complexes. In addition, activated platelets supply coagulation factors like FV that support the activation of prothrombin [36-40].

Patients with a defect in platelet function, which can be congenital or acquired, have a high risk of bleeding as a result of an impaired platelet activation per se and/or an impaired ensuing clot formation [41]. It is considered that an accurate estimation of the haemostatic potential is important for diagnosing and predicting the bleeding risk [42]. On method for this

is TG, which in PRP enables the investigation of interactions between platelets and coagulation factors in plasma, thus representing an assay that mimics such *in vivo* conditions [43]. In addition to exploring coagulation-dependent platelet disorders in pathophysiological conditions, the measurement of TG in PRP has been proposed as a potential tool for monitoring the effect of antiplatelet therapy [44]. It was shown that the use of aspirin in healthy individuals caused a prolonged lag time and time-to-peak (TTP). Moreover, the endogenous thrombin potential (ETP) was decreased in subjects using the glycoprotein (GP) IIb/IIIa antagonist abciximab [45,46]. Currently, the TG assay in PRP is regarded as a possibly important and additional tool to investigate individual functional characteristics of platelets under various clinical conditions [43].

PRINCIPLES OF THROMBIN GENERATION

Thrombin generation can be measured by the method of Calibrated Automated Thrombinography (CAT), which captures both the pro- and the anticoagulant parts of the coagulation cascade. The TG test gives insight into overall function of the haemostatic system [5,42]. The assay was originally designed to measure TG in platelet-poor plasma (PPP) and platelet-rich plasma (PRP), and later applied in whole blood (WB) after some adaptions [43,47,48]. After adding a procoagulant trigger, such as TF and phospholipids, the activity of the formed thrombin is registered over time using a specific fluorogenic substrate [5].

The conventional first-derivative thrombogram or TG curve (Figure 3) is characterized by an initiation phase (lag time) followed by a phase with formation of large amounts of thrombin (propagation), and a subsequent inhibitory phase of thrombin by natural anticoagulants [49]. Several thrombin parameters can be derived from the thrombogram: lag time, peak height, TTP, endogenous thrombin potential (ETP, area-under-the-thrombin-curve) and velocity index (VelIndex). The lag time is defined as the time needed to achieve $1/6^{th}$ of the concentration of thrombin present at peak height. The peak height represents the highest concentration of active thrombin formed during the experiment. The TTP is the time until the peak height is reached. The ETP is defined as the

area under the curve, and represents the total thrombin potential that a plasma sample can generate. The VI is the maximumslope of the propagation phase and is calculated as peak height / (TTP - lag time)[50].

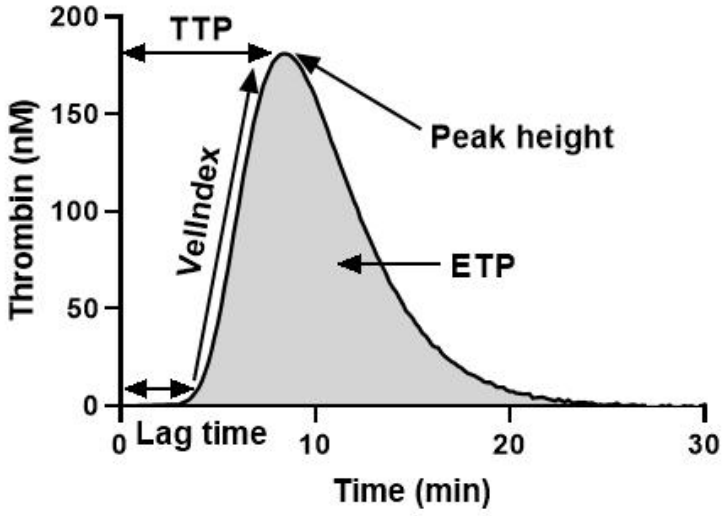

Figure 3. Thrombogram with various thrombin curve parameters. The thrombogram curve is usually quantified by the following parameters: lag time, time-to-peak (TTP), peak height, endogenous thrombin potential (ETP) and velocity index (VelIndex).

THE PRINCIPLE OF THROMBIN DYNAMICS

The first-derivative TG curve, corresponding to the proteolytically active thrombin concentration, is known to be the net result of prothrombin conversion and thrombin inactivation. The subsequent thrombin dynamics analysis is a computational method to derive parameters that quantify the pro- and anticoagulant mechanisms during TG, i.e. the prothrombin conversion and the thrombin inactivation, respectively [25,30]. The analysis takes into account the measured TG curve, the plasma fibrinogen level, which plays a modulating role, and the thrombin inhibitor levels of antithrombin and α_2M [51]. By quantifying the prothrombin conversion and thrombin inactivation capacities, more information is obtained

about pro- and anticoagulant processes that underlie TG.

The prothrombin conversion curve (Figure 4) shows the course of the conversion of prothrombin into thrombin over time. Several thrombin dynamics parameters can be defined from this curve: the total amount of prothrombin converted (PC_{tot}), the maximum rate of prothrombin conversion (PC_{max}), the thrombin-antithrombin complex formation (T-AT), and the thrombin-α_2M complex formation (T-α_2M). Additionally, the thrombin decay capacity (TDC) is quantified based on the plasmatic thrombin inhibitor levels independently of the TG curve [25,51,52].

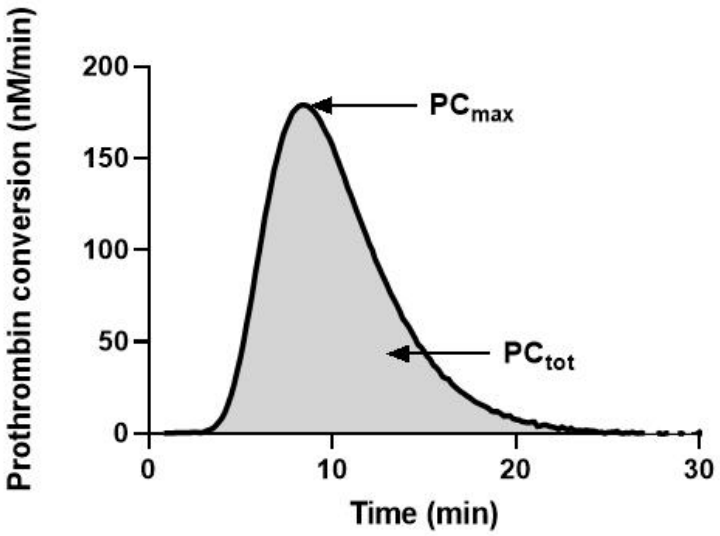

Figure 4. Prothrombin conversion curve with the derived parameters. An example of a prothrombin conversion curve is geiven with the total amount of prothrombin converted (PC_{tot}) and maximum rate of prothrombin conversion (PC_{max}).

THROMBIN GENERATION AND THROMBIN DYNAMICS IN CLINICAL SETTINGS

While the plasma levels of prothrombin fragment 1 + 2 and D-dimer indicate the *in vivo* ongoing etiological process of TG, the TG test can be used to detect an increased risk of

developing thrombosis or bleeding [33].

A high TG is usually associated with an increased risk of developing thrombosis. prese. A (venous) thrombosis may be caused by an excess of a coagulation factor(s) or a deficiency of a thrombin inhibitor(s), in conjunction with other eliciting factors like vessel wall, flow or platelet abnormalities, such in agreement with Virchow's triad. In a variety of studies, the plasma ETP and thrombin peak height were found to be elevated in carriers of virtually all thrombophilic defects, including FV and FII mutations and deficiencies of protein C, protein S or antithrombin [38,39]. Several epidemiological studies have further provided evidence that TG can be used to estimate the risk of venous thromboembolism (VTE), for both the occurrence of a first and a recurrent thrombotic event.

Conversely, a low TG is associated with an increased risk of bleeding. Excessive bleeding could occur due to the lack of coagulation factor(s) or by an excessive amount of an anticoagulant proteins, in combination with other bleeding enhancing factors like thrombocytopenia, low von Willebrand factor and vessel wall damage due to trauma[36].

In general, secondary thrombosis can be prevented by taking anticoagulants, and severe bleeding can be treated by the infusion of procoagulants, including plasma or coagulation factor concentrates [53,54]. As the TG assay is sensitive to all pro- and anticoagulant drugs, it thus could be used for monitoring patients requiring such agents.

The generation of thrombin is a dynamic process, and it is influenced by multiple underlying pro- and anticoagulant processes. An altered TG depends on the net effect of the conversion of prothrombin and the degradation of inhibitor-bound thrombin [25]. The thrombin dynamics method was previously developed to study the processes of prothrombin conversion and thrombin inactivation separately during thrombin generation. For this thesis pursuing recent publications, the question was asked how the thrombin dynamics method can be used in clinical settings and can help to resolve, why the TG changes in a certain way in specific patient groups. In general, a reduced thrombin generation potential in a patient population can have three main causes:

- the conversion of prothrombin into active thrombin is disturbed, and less thrombin is

generated as a result (e.g. because of FVIII deficiency in hemophilia A);

- the inactivation of thrombin is increased (e.g. because of treatment with heparins, which increases the efficiency of thrombin inhibition by antithrombin);
- the combination of both options stated above.

Vice versa, an increased thrombin generation potential can also have three causes:

- the conversion of prothrombin into active thrombin is overactive and more thrombin is generated as a result;
- the inactivation of thrombin is reduced (e.g. because of antithrombin deficiency);
- a combination of both options stated above.

Therefore, the thrombin dynamics method allows to provide increased mechanistic insight into changes in the coagulation system, when TG is increased, decreased or even unchanged between groups of patient and control subjects.

The addition of thrombin dynamics analysis to conventional TG has several advantages over using only TG. First, if a change in TG is detected in a certain patient population, this cannot be automatically pinpointed to a specific pro- or anticoagulant process in the coagulation system. The thrombin dynamics separation of pro- and anticoagulant processes allows us to explore whether the change in coagulability related to either process [52,55]. Second, the method can be used to distinguish whether seemingly similar TG curves are the result of similar underlying pro- and anticoagulant processes or whether the pro- and anticoagulant processes are changed in an oppositve way (e.g. cirrhosis patients) [30]. Table 1 shows an overview of previous clinical applications of the thrombin dynamics method. It has been used to help elucidate disturbances of the haemostatic system by exploring pro- and anticoagulant processes, as e.g. in patients with liver cirrhosis and patients after cardiopulmonary bypass [30,55,56]. Other studies have investigated the mechanisms of hypercoagulability in patient with antiphospholipid syndrome, asthma patients and subjects after strenuous exercise [52,57,58]. Other studies have contributed to our understanding of the differentiation and maturation of the coagulation system[31,59].

USE OF THROMBIN DYNAMICS FOR IN SILICO EXPERIMENTS

Thrombin dynamics analysis can furthermore be used for *in silico* modeling of the coagulation system, by varying the coagulant factor levels in a computational thrombin inactivation model. *In silico* experimentation was previously used to (1) test research hypotheses, (2) study the effect of multiple factors on TG simultaneously, or (3) predict the response of the coagulation system to an addition/deletion of coagulation factors. It was thus predicted by *in silico* experimentation that administration of prothrombin complex concentrates to prevent bleeding in patients with liver cirrhosis, can lead to a procoagulant state in these patients [30].

Table 1: Overview of previous clinical application of thrombin dynamics.

Study	Patient population	Conditions	Effects on thrombin dynamics	Effect on TG
Kremers et al., 2015	Blood group O	PPP and PRP at 1 and 5 pM TF	Lower PC_{tot} and TDC	Lower TG
Kremers et al., 2016	Cardiopulmonary bypass	PPP at 5 pM TF	Reduced prothrombin conversion Reduced thrombin inactivation	Lower TG
Kremers et al., 2016	Pediatrics	PPP at 1 and 5 pM TF	Reduced prothrombin conversion Reduced thrombin inactivation	Lower TG
Huskens et al., 2016	After strenuous exercise	PPP at 5 pM TF	Reduced PC_{tot} Increased PC_{max}	Lower ETP Higher peak height
Bazan-Socha et al., 2016	Asthma patients	PPP at 5 pM TF	Higher PC_{tot} and PC_{max} Lower TDC	Higher TG
Kremers et al., 2017	Liver cirrhosis patients	PPP at 5 pM TF	Reduced prothrombin conversion Reduced thrombin inactivation	Rebalanced TG
Kremers et al., 2018	Antiphospholipid syndrome patients	PPP at 1 and 5 pM TF	Elevated PC_{max}	Higher TG (peak height)
Beattie et al., 2020	Severe pediatric liver disease	PPP at 5 pM TF	Reduced prothrombin conversion Reduced thrombin inactivation	Lower TG

PPP: Platelet poor plasma; PRP: platelet rich plasma; TF: tissue factor; PC_{tot}: total amount of prothrombin converted; TDC: thrombin decay capacity; TG: thrombin generation; PC_{max}: maximum prothrombin conversion rate; ETP: endogenous thrombin potential.

OVERVIEW OF THIS THESIS

The goal of this thesis is to further explore the clinical meaning of the different parameters of TG and thrombin dynamics in blood plasma, such as set out in **Chapter 1**. Investigations concern the contribution of coagulation factors and platelets to each thrombin dynamics parameter. In **Chapter 2**, we investigate effects of the prothrombin complex factors on the dynamics of thrombin generation and inactivation. The study concentrates on how the prothrombin conversion is affected by prothrombin, FX and antithrombin, and on how the thrombin inactivation depends on antithrombin and fibrinogen. It is also aimed to establish reference values of thrombin dynamics in platelet-poor plasma to provide guidance values for clinically 'normal' and 'abnormal' parameter values. In **Chapter 3**, the investigations concentrate on the dynamics of TG in HIV-infected patients. Moreover, the effect is examined of different HIV treatment strategies on coagulation, such as abacavir, which is associated with an increased risk for developing thrombosis. **Chapter 4** concerns a study on thrombin dynamics using TG parameters, such as measured with the frequently used ST Genesia roboting machine. In **Chapter 5**, we examine how the platelet count influences the TG and thrombin dynamics parameters in platelet-rich plasma. Main question is how an increase in the platelet count alters the TG and the rate of prothrombin conversion. In addition, the reference ranges for TG and thrombin dynamics in platelet-rich plasma are established. The final **Chapter 6** provides a general discussion of the key results of this thesis, given the recent literature.

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Chapter 2

Deciphering the coagulation profile through the dynamics of thrombin activity

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ABSTRACT

Thrombosis has proven to be extremely difficult to predict. Measuring the generation of thrombin is a very sensitive method to detect changes in the hemostatic system. We developed a method based on the generation of thrombin to further fingerprint hemostasis, which we have named thrombin dynamics.

Via this method we are able to exactly measure the prothrombin conversion and thrombin inactivation, and any change in the coagulation cascade will be reflected in these two processes. In the current study we analyzed the importance of the members of the prothrombin complex on the dynamics of thrombin activation and inactivation. We show that prothrombin conversion is predominantly influenced by factor X and antithrombin, which will provide essential insights in complex thrombosis-related diseases, such as liver cirrhosis and kidney failure.

INTRODUCTION

Thrombin is the key enzyme in the coagulation cascade and converts fibrinogen into a fibrin network. The thrombin generation (TG) test measures the amount of thrombin that is generated in plasma in response to a tissue factor stimulus[1]. TG is a widely used method to screen for hyper- and hypo-coagulability[2], as increased TG is associated with thrombosis, and vice versa, reduced TG is related to bleeding[2-8]. Additionally it is often used to assess therapeutic strategies, both in research[9,10] and in the clinic[11,12]. It is a global coagulation assay and subsequently, a deviant TG profile cannot be immediately attributed to a specific coagulation defect[1,2] and further testing is required.

The thrombin generation describes the amount present in clotting plasma at each time point during the measurement. The thrombin concentration depends on two main underlying processes: the production of thrombin (prothrombin conversion) and inactivation of thrombin[13]. A reduction of TG can be caused by lower activation of the prothrombin conversion or increased thrombin inhibition. Recently, we developed a method called thrombin dynamics analysis to study the processes that underlie thrombin generation in more detail[14]. In this method, we quantify prothrombin conversion and thrombin inactivation from TG data, allowing these processes to be studied independently from each other. The rate of thrombin inactivation is predicted with an algorithm based on the plasma antithrombin (AT), α_2Macroglobulin (α_2M) and fibrinogen level[14]. Subsequently, the prothrombin conversion curve can be extracted from the thrombin generation curve. From this prothrombin conversion curve, the peak value and the area-under-the-curve are quantified (Figure 1), respectively representing the maximum rate of the prothrombinase complex (PC_{max}) and the total amount of prothrombin converted throughout the measurement (PC_{tot}). The amount of thrombin-antithrombin (T-AT) and thrombin-α_2Macroglobulin (T-α_2M) complexes formed during the experiment are quantified. The thrombin inactivation capacity (TDC) is calculated independent from the TG curve and depends solely on the AT, α_2M and fibrinogen level of a plasma sample.

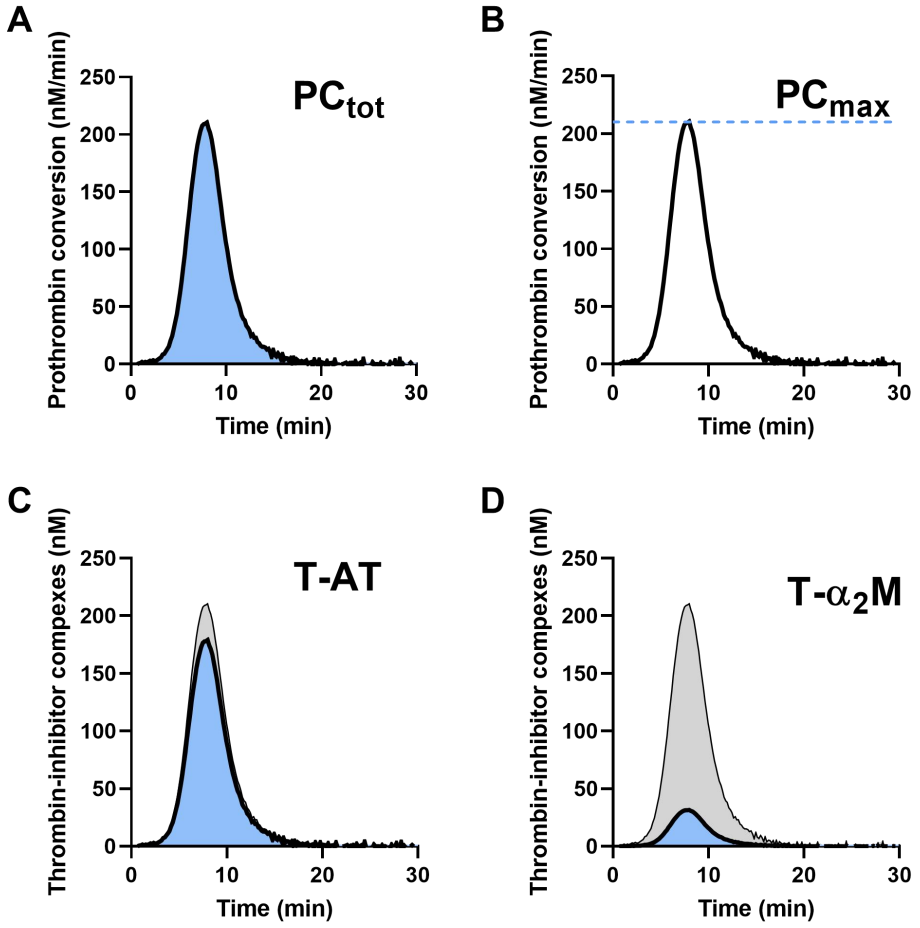

Figure 1: Illustration of the quantification of thrombin dynamics parameters. (A) The total amount of prothrombin converted (PC_{tot}) is quantified as the area under the curve of the prothrombin conversion curve. (B) The maximum rate of prothrombin conversion (PC_{max}) is defined as the peak of the prothrombin conversion curve. (C-D) The total amount of prothrombin converted during TG equals the total amount of thrombin-inhibitor complexes formed (gray area). This is split into thrombin-antithrombin complex formation (T-AT; C) and thrombin-α_2M formation (T-α_2M; D).

The dynamics of thrombin generation have been studied in multiple clinical settings over the past years to study the balance between pro-and anticoagulant mechanisms, and to

perform *in silico* experimentation to generate hypotheses[14-22]. Recently, questions have started to emerge about the influence of individual coagulation factor levels on the parameters of prothrombin conversion and thrombin inactivation. It is of interest to study the contribution of specific coagulation factors to the individual parameters in order to further fingerprint coagulation. This allows the better interpretation of *in silico* results and the generation of new working hypotheses based on the *in silico* work. Another important question that needs to be addressed is when the novel parameters should be considered abnormal. Until now, reference ranges were not available for prothrombin conversion and thrombin inactivation parameters, which makes it difficult to interpret the assays results clinically when a study is performed in a clinical setting. Especially in the case of *in silico* experimentation on clinical data, reference values are an important tool to define what is considered normal and what is not.

Thrombin is the last enzyme of the coagulation cascade which converts fibrinogen into fibrin thereby changing the liquid blood into a solid compound. Any change in the coagulation factors will have an effect on the generation and activity of thrombin. In the current study, we investigated the effect of individual coagulation factors mostly belonging to the prothrombinase complex on the dynamics of thrombin activity using thrombin generation, prothrombin conversion and thrombin inactivation as read out.

METHODS

Sample collection

Our study protocols were evaluated by the local medical ethical boards (Medical Ethical Committee of Maastricht University Medical Center or Erasmus Medical Center Rotterdam). All research was performed in accordance with the relevant guidelines and regulations, and all volunteers gave full informed consent according to the Helsinki declaration. The study population consisted of 122 healthy adult individuals, aged 18-65 years. None of the participants used oral anticoagulant or anti-platelet drugs for at least two weeks, had a history of thrombosis or bleeding. Additionally, 8 haemophilia A patients were included. Blood was collected into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System/Greiner). Platelet-poor plasma (PPP) was obtained by double centrifugation at 2630g for 10 min and stored at -80°C until further use.

Materials

Hepes buffers containing 5 mg/ml or 60 mg/ml bovine serum albumin were used to dilute the reagents or substrates, respectively, as described before[13]. Bovine serum albumin and unfractionated heparin and were purchased at Sigma-Aldrich (Darmstadt, Germany). The chromogenic thrombin substrate, S2238, was synthesized in house (Synapse Research Institute, Maastricht, the Netherlands)[23]. Bovine thrombin and bovine antithrombin were purified according to the protocols of Church et al. and Thaler et al. (Synapse Research Institute, Maastricht, the Netherlands)[24,25].

Coagulation factor determinations

All coagulation factor levels except α_2-macroglobulin (fibrinogen, FII, FV, FX, and antithrombin) were determined on the STA-R Evolution analyzer (Diagnostica Stago, Asnières, France). Fibrinogen levels were measured with the Claus assay. Functional α_2M levels were determined in house as previously described (Synapse Research Institute, Maastricht, the Netherlands)[14].

Thrombin generation

Calibrated Automated Thrombinography (CAT) was performed as previously described. PPP reagent low and PPP reagent, corresponding to 1 and 5 pM tissue factor (Diagnostica Stago, Asnières, France) were used according to the manufacturers description[13]. The results were analyzed with the Thrombinoscope software (Thrombinoscope, Maastricht, the Netherlands). The TG curves were used to perform additional computational analysis to extract prothrombin conversion curves[14].

Thrombin dynamics

The TG curve is the net result of prothrombin conversion and thrombin inactivation and therefore, the prothrombin conversion curve can be calculated from a TG curve.

Thrombin inactivation was predicted by the previously described and validated computational model[14,19,26,27]. This model consists of a set of ordinary differential equations, which describe the rate of thrombin inactivation in time based on the plasma AT, α_2M and fibrinogen level and the free thrombin concentration at each point in time (eq. 1-3).

Eq. 1 $d(T\text{-}AT)/dt = k_{AT} \cdot [AT]_t \cdot [T_{free}]_t$

Eq. 2 $d(T\text{-}\alpha_2M)/dt = k_{\alpha 2M} \cdot [\alpha_2M]_t \cdot [T_{free}]_t$

Eq. 3 $-d(T_{free})/dt = k_{AT} \cdot [AT]_t \cdot [T_{free}]_t + k_{\alpha 2M} \cdot [\alpha_2M]_t \cdot [T_{free}]_t$

The amount of thrombin that is free in solution (T_{free}) depends on the amount of thrombin substrate that is present, and rate constants for the inactivation of thrombin by antithrombin (k_{AT}) and α_2-macroglobulin ($k_{\alpha M}$) are dependent on the plasma fibrinogen level, as described in more detail elsewhere[14].

At any moment during the course of the TG process, the TG curve is the net result of prothrombin conversion and thrombin inactivation. Therefore, the course of prothrombin conversion (d(P)/dt) can be calculated from the TG curve ([T]t) and the inactivation rate of thrombin at a specific thrombin concentration (d(T-inh)/dt) (Eq. 4). With the previously described model for thrombin inactivation we can calculate the thrombin inactivation rate at

each time point during TG (Eq. 5).

Eq. 4 $d(T)/dt = -d(P)/dt - d(T\text{-}inh)/dt$

Eq. 5 $-d(P)/dt = d(T)/dt + k_{AT} \cdot [AT]_t \cdot [T]_t + k_{\alpha 2M} \cdot [\alpha_2M]_t \cdot [T]_t$

Thrombin inactivation can be quantified by the thrombin decay capacity which is the pseudo-first order decay constant for thrombin that combines the overall effect of thrombin inactivation by AT and α_2M. The prothrombin conversion curve is quantified by its area-under-the-curve, which translates to the total amount of prothrombin converted (PC_{tot}) throughout the TG experiment, and the peak height of the prothrombin conversion curve, which is the maximum prothrombin conversion rate (i.e. the maximum activity of the prothrombinase complex; PC_{max}).

Statistical analysis

Statistical analysis was performed using Graphpad Prism (version 8, San Diego, USA). Reference ranges were determined as the 2.5th and 97.5th percentile values of the healthy subjects dataset variables. Data was presented as the median ± interquartile range. Statistical significance was determined by ANOVA analysis with Bonferroni correction or the Mann-Whitney test, dependent on the number of groups that needed to be analyzed. Dose-response effects were investigated using linear correlation. Correlations were calculated as the Pearson correlation coefficient in the healthy subject sample data. A p-value below 0.05 was considered statically significant.

RESULTS

Even though the thrombin dynamics method has shown its added value to thrombin generation data in several clinical studies in the past years, the meaning of each parameter in the wider context of coagulation has yet to be investigated[14-22]. In this study, we

investigated the effect of four major coagulation factors (prothrombin, FV, FX, and antithrombin) on five thrombin dynamics parameters (PC_{tot}, PC_{max}, T-AT, T-α_2M, and TDC). We chose to study prothrombin, FV and FX because they are components of the prothrombinase complex, converting prothrombin into active thrombin. In addition, antithrombin was studied because it is the most important natural thrombin inhibitor. The role of each individual coagulation factor was investigated by performing dose-response measurements in plasma deficient in that specific factor and by performing correlation analysis in a group of healthy subjects (n=122).

The effect of plasma prothrombin level on the dynamics of thrombin generation

We first investigated the effect of prothrombin on thrombin generation, prothrombin conversion and thrombin inactivation. The plasma prothrombin level has a strong dose-dependent effect on thrombin generation (Supplementary figure 1).

Below the threshold of 20%, prothrombin shortens the lag time and time-to-peak dose-dependently, and the prothrombin level correlates almost perfectly with ETP and peak at 5 pM TF ($R^2 = 0.972$ and $R^2 = 0.971$, respectively). We used thrombin dynamics analysis to further fingerprint the effect of the prothrombin level on thrombin generation. As expected, we found that prothrombin dose-dependently increases prothrombin conversion (Figure 2A-F). Not only PC_{tot}, but also T-AT and T-α_2M are linearly correlated with the plasma prothrombin level (all $R^2 > 0.973$) and PC_{max} increases with increasing prothrombin levels, which is most pronounced at high TF concentrations.

In healthy subjects, both PC_{tot} and T-AT are correlated to the plasma prothrombin level at 1 pM TF ($R^2 = 0.136$ and $R^2 = 0.131$, respectively with $p<0.0001$) and at 5 pM TF ($R^2 = 0.183$ and $R^2 = 0.195$, respectively with $p<0.0001$). TDC does not depend on prothrombin in *in vitro* dose-response experiments (Supplementary figure 2), nor in healthy subjects.

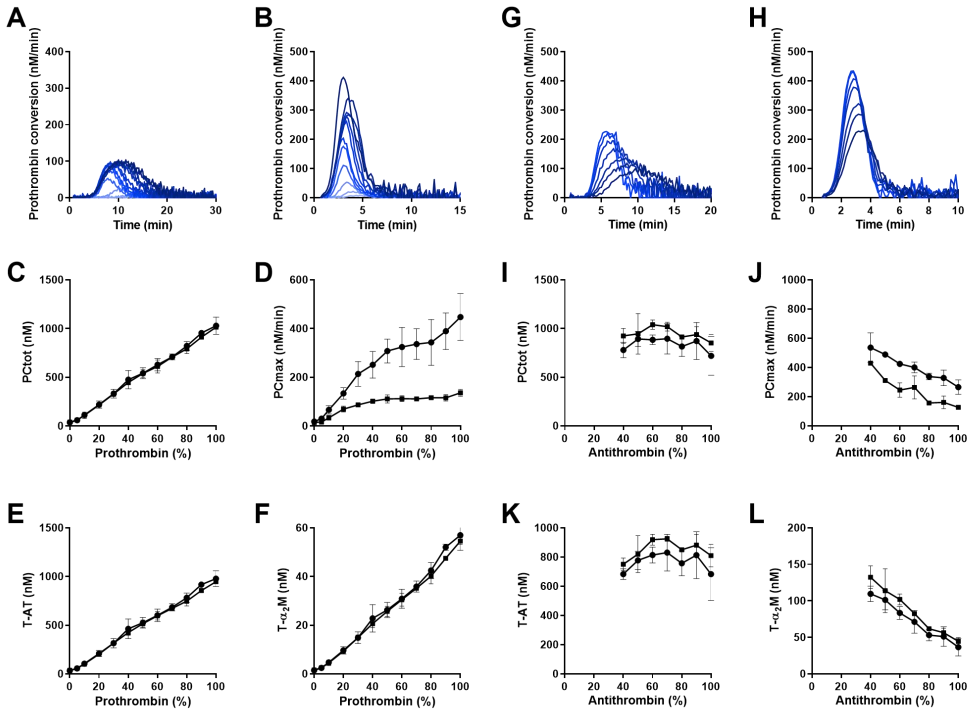

Figure 2: The effect of prothrombin and antithrombin on the dynamics of thrombin generation. **(A-F)** Prothrombin deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% prothrombin. Prothrombin conversion curves at 1 (A) and 5 pM TF (B) are shown (0-100% prothrombin from bottom to top) and thrombin dynamics parameters PC_{tot} (C), the PC_{max} (D), T-AT complexes (E) and T-α_2M complexes (F) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). **(G-L)** Antithrombin deficient plasma was mixed with pooled normal to achieve plasma concentrations of 40, 50, 60, 70, 80, 90, and 100% antithrombin. Prothrombin conversion curves at 1 (G) and 5 pM TF (H) are shown (0-100% antithrombin from top to bottom and thrombin dynamics parameters PC_{tot} (I), the PC_{max} (J), T-AT complexes (K) and T-α_2M complexes (L) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). The average results of 3 experiments are shown as the mean ± SD.

The effect of plasma AT level on the dynamics of thrombin generation

Secondly, we quantified the effect of the natural anticoagulant antithrombin on thrombin generation, prothrombin conversion and thrombin inactivation. Thrombin generation was measured above an antithrombin level of 40% (Supplementary figure 1) and thrombin dynamics parameters were quantified (Figure 2G-L). Due to experimental limitations, thrombin generation cannot be measured in plasma samples containing less antithrombin because then prothrombin levels exceed antithrombin levels, causing ongoing thrombin generation and subsequent substrate depletion. Antithrombin significantly prolongs the thrombin generation lag time and time-to-peak is inversely correlated with the ETP and peak ($p<0.001$). We used thrombin dynamics analysis to investigate whether antithrombin only influences the inactivation of thrombin, or prothrombin conversion as well. Predominantly the maximum rate of prothrombin conversion (PC_{max}) was attenuated by antithrombin (Figure 2G-L). We also found that the amount of thrombin-α_2M complexes formed during thrombin generation was reduced at increasing antithrombin concentrations ($p<0.001$). In healthy subjects, we were able to confirm that the AT level was significantly correlated to the T-α_2M level at 1 pM TF ($R^2=0.132$ with $p<0.0001$) and 5 pM TF ($R^2=0.158$ with $p<0.0001$). In addition, the thrombin decay constant strongly depends on the plasma AT level in the dose-response measurements (Supplementary figure 2) and in healthy subjects ($R^2=0.537$, $p<0.0001$).

The effect of plasma FV level on the dynamics of thrombin generation

Factor V is one of the prominent members of the prothrombinase complex and we tested its role in thrombin generation, prothrombin conversion and thrombin inactivation. FV dose-dependently shortened the lag time and time-to-peak, especially below 20% FV (Supplementary figure 3).

Additionally, FV increased the TG peak height and ETP dose-dependently ($p=0.004$ and $p<0.001$). Using thrombin dynamics analysis, we found that the FV increases thrombin generation at low TF levels by stimulating the production of thrombin ($PC_{tot,}$ $p=0.0023$),

which subsequently increases the formation of T-AT and T-a_2M (p=0.048 and p=0.0045, respectively; Figure 3A-F).

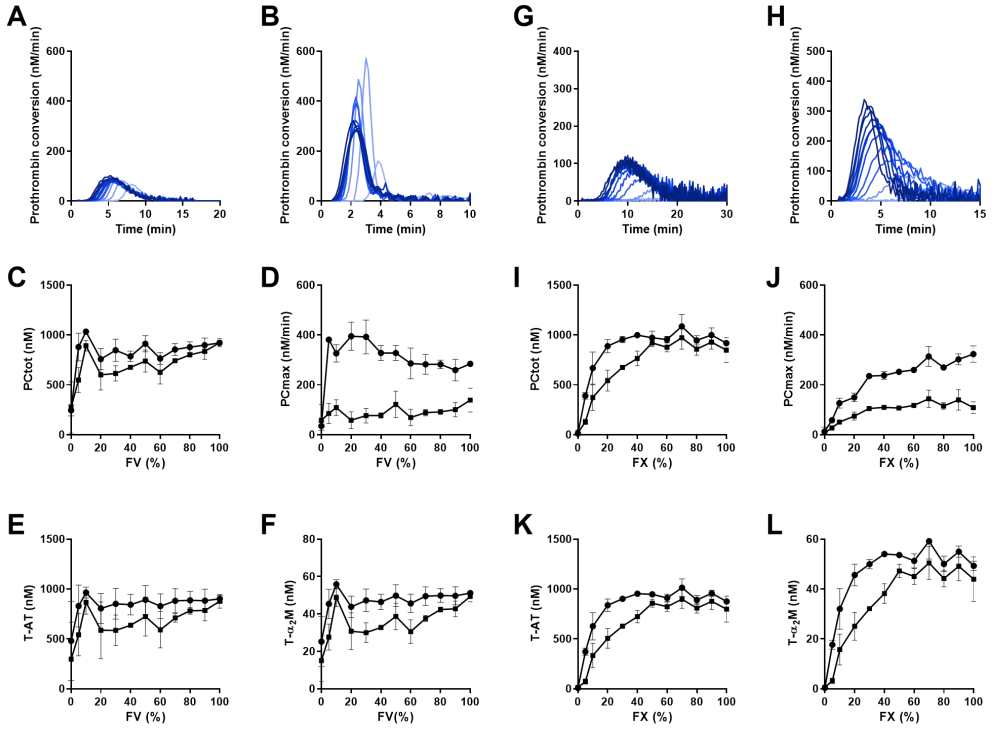

Figure 3: The effect of FV and FX on the dynamics of thrombin generation.(A-F) FV deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% FV. Prothrombin conversion curves at 1 (A) and 5 pM TF (B) are shown (0-100% FV from bottom to top) and thrombin dynamics parameters PC_{tot} (C), the PC_{max} (D), T-AT complexes (E) and T-α_2M complexes (F) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). **(G-L)** FX deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% FX. Prothrombin conversion curves at 1 (G) and 5 pM TF (H) are shown (0-100% FX from bottom to top and thrombin dynamics parameters PC_{tot} (I), the PC_{max} (J), T-AT complexes (K) and T-α_2M complexes (L) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). The average results of 3 experiments are shown as the mean ± SD.

At 5 pM TF, FV did not significantly affect any of the thrombin dynamics parameters. In contrast, in healthy subjects FV did not show a significant correlation with any of the thrombin dynamics parameters. Furthermore, the thrombin decay constant did not depend on the plasma FV level (Supplementary figure 2), and also in the 122 healthy subjects, FV did not correlate with the thrombin decay constant.

The effect of plasma FX level on the dynamics of thrombin generation

Factor X levels were measured and studied for their influence on thrombin. FX dose-dependently increased the thrombin generation peak height and ETP ($p<0.001$ and $p=0.0004$) and shortens the lag time and TTP. Thrombin dynamics analysis revealed that TG increases because of an increase of PC_{tot}, T-AT and T-α_2Min the lower range of FX (Figure 3 G-L), whereas the thrombin decay constant was not affect by the plasma FX level (Supplementary figure 2). Factor X levels above 40% did not increase PC_{tot}, T-AT and T-α_2M any further and resulted in a plateau (all $p<0.0001$). Additionally, PC_{max} increased dose-dependently with the FX level ($p<0.0001$), most pronouncedly at 5 pM TF. In healthy subjects, the plasma FX level correlates significantly with PC_{tot} at 1 and 5 pM TF ($R^2=0.101$ and $R^2=0.142$ with $p<0.0005$) and T-AT at both 1 and 5 pM TF ($R^2=0.102$ and $R^2=0.151$ at $p<0.0001$), but not T-α_2M.

Reference values for thrombin dynamics

In order to define normal values for thrombin dynamics parameters, we measured thrombin generation and thrombin dynamics in 122 healthy subjects at 1 and 5 pM TF (Supplementary table 1). We defined the reference ranges as the 2.5^{th} and 97.5^{th} percentile for the thrombin dynamics parameters in the whole group of healthy subjects (n= 122), which are depicted in Figure 4 as grey boxes. The total amount of prothrombin converted (PC_{tot}) ranged from 693 to 1344 and from 746 to 1335 for TG triggered with 1 and 5 pM TF, respectively. The maximum prothrombin conversion rate (PC_{max}) ranged from 109 to 415 for 1 pM TF, and from 153 to 474 for 5 pM TF. Thrombin-antithrombin complexes ranged from 667 to 1283 for 1 pM TF and from 729 to 1279 for 5 pM and thrombin-α_2M ranged

from 16 to 63 both for 1 and 5 pM TF. The thrombin decay capacity ranged from 0.633 min^{-1} to 1.002 min^{-1} with a median value of 0.816 min^{-1}.

The dynamics of thrombin generation in men vs. women and the effect of oral contraceptives

As oral contraceptives have been shown to affect coagulation and the generation of thrombin via an effect on the protein C pathway we studied possible differences between men, women with/without OC in more detail. Figure 4 shows the average thrombin generation and prothrombin conversion curves for the subset of men (n=60), women without oral contraceptives (OC; n=47), and women with OC (n=15). As expected from previous studies, thrombin generation was significantly higher in women than in men (ETP +8%, p=0.043) when measured at 1 pM TF. Within the group of women, the ETP was significantly higher in women using OC (+25%, p<0.01) compared to women without OC. No difference was found between men and women without OC. To understand these differences in TG, we studied thrombin dynamics parameters in men, women without OC, and women with OC. We found that PC_{tot} (+14.4% and +9.5% for 1 and 5 pM TF), PC_{max} (36.9+% and +46.1% for 1 and 5 pM TF), and T-AT (+14.3% and +9.5% for 1 and 5 pM TF) were significantly elevated in women with OC compared to women without OC. T-α_2M formation was unaffected by OC use. No differences were found between men and women without OC.

The main determinants of thrombin dynamics parameters

We investigated in more detail possible combined effects of our input coagulation factors. Therefore, 3D plots were drawn in order to depict the relationship between each thrombin dynamics parameters and its two most important influencing coagulation factors. We found that PC_{tot} is mainly dependent on prothrombin and FX levels, and low levels of either coagulation factor lead to lower prothrombin conversion (Figure 5A). PC_{max} is mainly dependent on prothrombin and FX (Figure 5B). Thrombin-antithrombin formation is mostly

dependent on the levels of prothrombin and FX as high prothrombin or FX levels are associated with high amounts of T-AT complexes formed (Figure 5C). Figure 5D shows that thrombin-$\alpha_2$macroglobulin formation is high when as expected α_2M levels are high, and T-α_2M formation is low when α_2M is low. The thrombin decay capacity mainly depends on antithrombin and fibrinogen levels, and high TDC is associated with high antithrombin levels and low fibrinogen levels (Figure 5E).

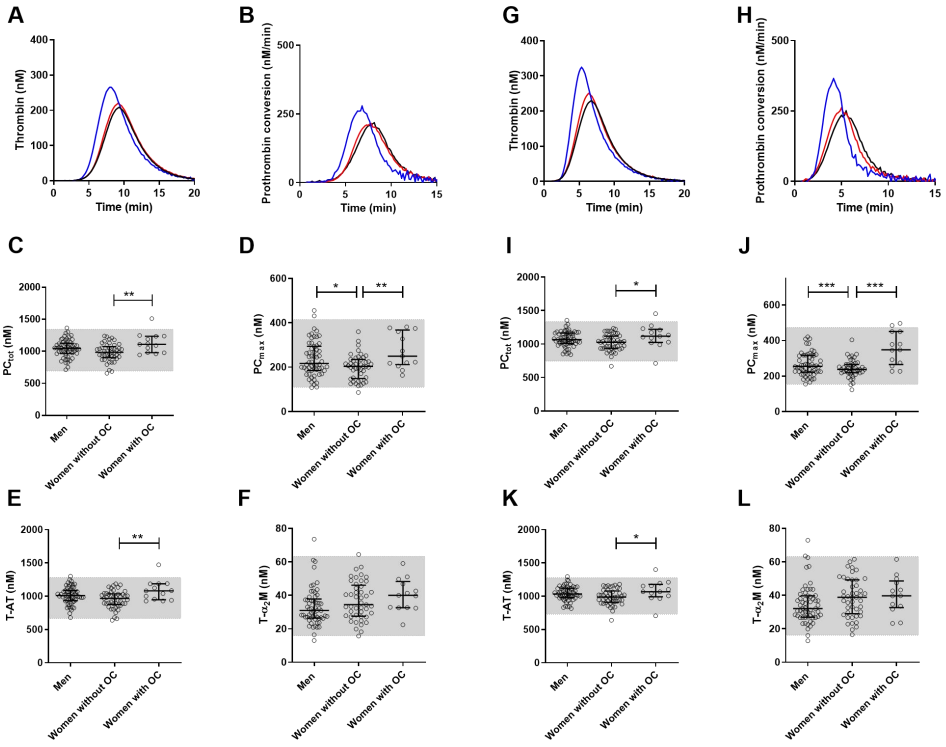

Figure 4: Reference values for thrombin dynamics parameters determined in 122 healthy subjects. Thrombin generation and thrombin dynamics were determined at 1 pM TF (A-F) and 5 pM TF (G-L). Average thrombin generation curves at 1 and 5 pM TF (A and G) were calculated for 3 groups: men (black), women without oral contraceptives (OC; red) and women with OC (blue). Average prothrombin conversion curve at 1 and 5 pM TF were calculated for the same groups (B and H). Reference ranges were determined for thrombin dynamics parameters at 1 and 5 pM TF: PC_{tot} (C and I), PC_{max} (D and J), T-AT (E and K) and T-α_2M (F and L). Reference ranges are depicted as grey

boxes, dots show individual values and the lines indicate the median ± interquartile range. Statistical significance was tested by ANOVA with Bonferroni correction was indicated as *p<0.05, **p<0.001, and ***<p<0.001.

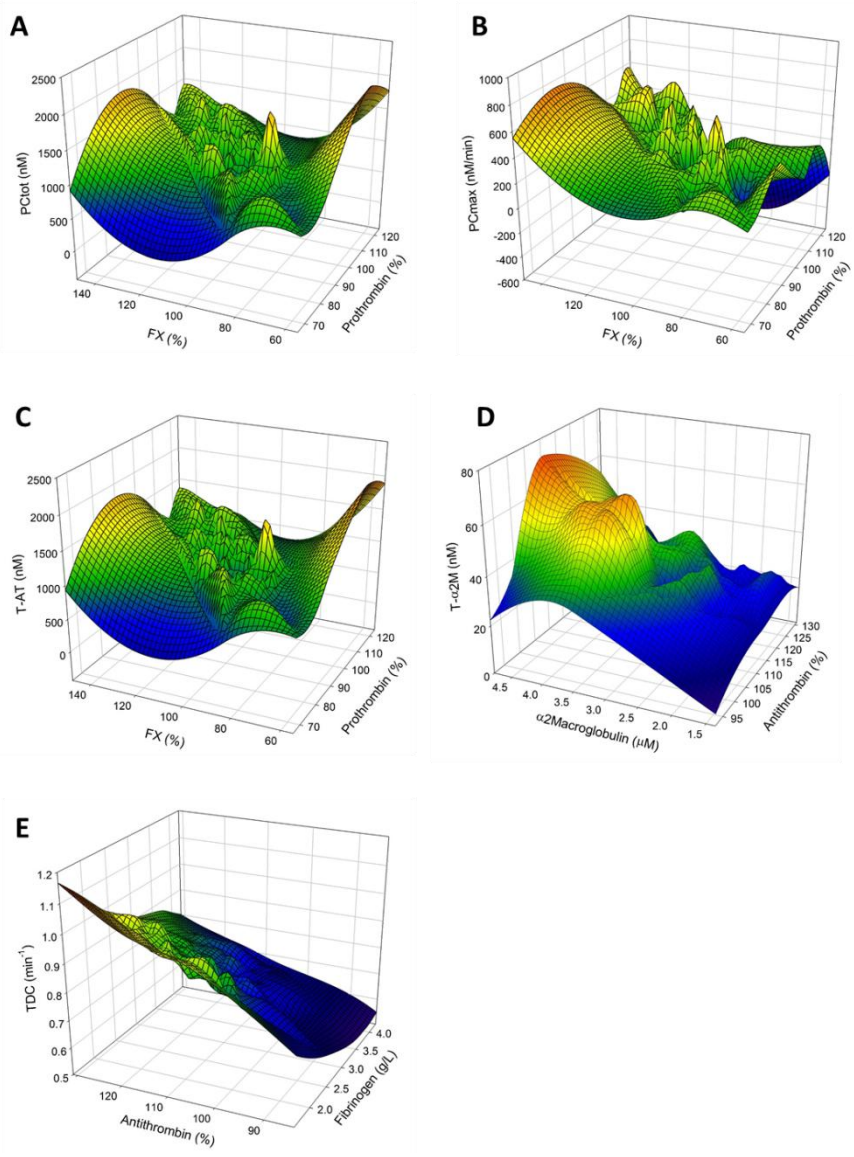

Figure 5: The main determinants of each thrombin dynamics parameter in 122 healthy subjects. (A) The influence of prothrombin and FX levels on the PC_{tot}, (B) of prothrombin and FX levels on the PC_{max}, (C) FII and FX levels on T-AT formation, (D) AT and α_2M levels on T-α_2M formation and (E) AT and fibrinogen levels on the TDC. 3D plots show the overall trend of the data as a color coded mesh, ranging from blue (low values) through green and yellow to red (high values).

Prothrombin conversion and thrombin inactivation in haemophilia A

To illustrate the thrombin dynamics method further, we measured thrombin generation, prothrombin conversion and thrombin inactivation in 8 hemophilia A patients. Figure 6 A-B shows the average thrombin generation curve and prothrombin conversion curve at 1 pM TF in hemophilia A patients and 60 healthy male controls. Both the rate of prothrombin conversion and the amount of prothrombin converted is reduced in hemophilia. Subsequently. T-AT and T-α_2M complex formation were significantly lower in patients compared to controls.

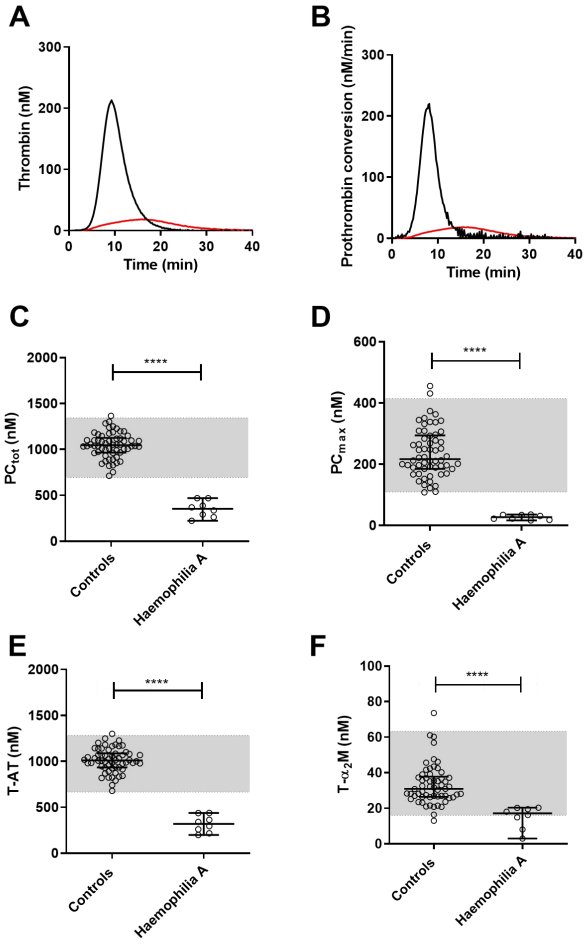

Figure 6: Prothrombin conversion and thrombin inactivation in haemophilia A. Thrombin generation and thrombin dynamics were determined at 1 pM TF in 60 (male) controls and 8 hemophilia A patients. Average thrombin generation curves (A) were calculated for the controls (black) and hemophilia patients (red). The average prothrombin conversion curve at 1 pM TF were calculated for the same groups (B). Prothrombin conversion was quantified as PC_{tot} (C) and PC_{max} (D), and thrombin inactivation was measured by T-AT (E) and T-α_2M (F). Reference ranges are depicted as grey boxes, dots show individual values and the lines indicate the median ± interquartile range. Statistical significance was tested by Mann-Whitney test and indicated as ****<p<0.0001.

DISCUSSION

Thrombin is the central enzyme in coagulation by converting fibrinogen into fibrin thereby forming a clot. Any change in the coagulation cascade will be depicted in the generation and activity of thrombin. Thrombin generation is a sensitive method to investigate these changes, but this method will not provide detailed reasons why the coagulation behaves differently in certain situations. In the current study, we hypothesized that each specific coagulation factor has a 'fingerprint', a specific influence on thrombin dynamics, which is initially revealed as a deviation in the TG profile. Thrombin dynamics is a method that goes further into detail quantifying the prothrombin conversion and the thrombin decay. By using thrombin dynamics we were able to pinpoint how factor II, V, X, antithrombin and α_2M affect the generation of thrombin.

Changes in thrombin generation are caused by specific changes in the underlying processes of prothrombin conversion and thrombin inactivation. Prothrombin conversion is not only influenced by procoagulant factors prothrombin and FX, but also by antithrombin. The total amount of prothrombin conversion is dependent on the available amount of prothrombin in the plasma, which is intuitively logical. In contrast, the maximum velocity of prothrombin conversion is not only dependent on the plasma level of prothrombin and FX, but also on the antithrombin level. This finding is in line with the data of Cvirn et al that show that an increase of antithrombin levels reduces the amount of prothrombin fragment 1+2 formation[28]. Furthermore, according to the laws of enzyme kinetics, the prothrombin level is a rate limiting factor for the maximum prothrombin conversion velocity because it is the substrate for the prothrombinase complex[29]. The other (theoretical) rate limiting factor is the concentration of the enzyme of the prothrombinase complex, FX, which is confirmed by our experimental results. Therefore, a lower prothrombin or FX, and especially the combination of lower levels of both, result in a reduced PC_{max}. Indeed, in a previous study we found a reduction of PC_{max} in patients on vitamin K antagonists[22].

The formation of thrombin-antithrombin and thrombin-α_2M complexes depends on the plasma level of the respective inhibitor and the prothrombin level. Low prothrombin levels

and subsequently low prothrombin conversion lead to low T-AT and T-α_2M formation, simply because thrombin needs to be available to be inhibited. Nevertheless, the antithrombin and α_2M have a large effect on the amount of T-AT and T-α_2M formed, respectively, and the ratio between the two types of enzyme-inhibitor complexes. In patients treated with heparin, whose anticoagulant effect is mediated through the facilitation of thrombin inhibition by AT[30], the balance between T-AT and T-α_2M shifts towards the formation of T-AT complexes. On the contrary, in subjects with low AT levels or high α_2M levels, such as liver cirrhosis patients and young children, the balance between T-AT and T-α_2M has been shown to shift in favor of T-α_2M complexes[21,27].

We have used thrombin dynamics analysis to study the process of prothrombin conversion and thrombin dynamics in multiple clinical settings. Thrombin generation shows a deviation in certain patients groups, but the deviation cannot be pinpointed to a specific target process in the coagulation cascade. We used thrombin dynamics analysis to provide more information of the mechanism of disease. Additionally, thrombin dynamics can be used to perform *in silico* experimentation, because of the computational model for thrombin inactivation that lies at its basis. This allows us to test research hypothesis *in silico* as illustrated below with several examples. To define what is abnormal we defined normal values for thrombin dynamics parameters PC_{tot}, PC_{max},T-AT, T-α_2M, and TDC and compared these results to previously acquired thrombin dynamics data. Supplementary table 2 shows an overview of previous clinical thrombin dynamics studies and how the respective patient populations relate to the newly established normal ranges[14-22].

Liver cirrhosis causes disturbed blood coagulation due to the reduced production of pro- and anticoagulant factors, such as prothrombin, FV, FX, and antithrombin[31-33]. As a result, cirrhosis patients suffer from both bleeding (eg bruising, ruptured esophageal varices) and thrombosis (eg deep venous thrombosis, pulmonary embolism)[34,35]. Nevertheless, routine clinical tests such as the prothrombin time indicate increased bleeding risk, whereas thrombin generation correlates better with the hemostatic situation in cirrhosis patients[31]. We found that prothrombin and antithrombin levels were significantly reduced to

respectively 43% and 39% in severe liver cirrhosis[21]. In contrast, α_2Macroglobulin was increased up to 2-fold resulting in decreased PC_{tot}, T-AT and TDC values in thrombin dynamics analysis. In addition, T-α_2M levels are elevated compared to healthy subjects and newly established reference values.

Children hardly ever suffer from thrombosis[36] and thrombin generation is reduced in children compared to adults[37-39]. We recently investigated the mechanism behind this change and found that prothrombin conversion (PC_{tot} and PC_{max}) is lower in children compared to adults[20]. Additionally, the balance between antithrombin and α_2M for the inhibition of thrombin shifts more towards α_2M. Nevertheless, liver disease in children causes striking similarities to the changes in adults[40]. However, the impressive reduction of prothrombin conversion provides an explanation for the diminished risk of pediatric thrombosis.

Hemophilia A is a genetic disorder that causes low or undetectable levels of the pro-coagulant FVIII and subsequently, patients have a severe risk of bleeding. Thrombin generation, prothrombin conversion and thrombin inactivation are reduced in hemophilia[6,41], and has been used in the past to estimate a patients risk of bleeding[42]. A novel treatment approach in hemophilia is to attack the anticoagulant pathway, in an attempt to bring the pro- and anticoagulant process in balance[11]. We used the thrombin dynamics method and *in silico* simulations to investigate the effect of antithrombin-targeting in haemophilia A patients on thrombin dynamics and thrombin generation (unpublished work). A 50% reduction of antithrombin could increase thrombin generation peak height in haemophilia A patients by 80%. However, the variation in effect between patients was large, depending on their initial thrombin generation profile, and therefore, pre-therapeutic dose adjustment using the thrombin dynamics method might be interesting.

The thrombin generation method and subsequently the quantification of prothrombin conversion and thrombin inactivation have some limitations associated with in vitro testing. One limitation is the absence of platelets in the measurements in the current work. However, thrombin generation can also be measured in platelet rich plasma[43]. Furthermore, we have

recently published that thrombin dynamics analysis can also be performed in platelet rich plasma and that the measurement parameters are sensitive to the platelet number[44]. Another limitation is the lack of the vessel wall, which plays a role in in vivo coagulation. In thrombin generation, this can be mimicked partially using soluble thrombomodulin[1]. Therefore, the results of thrombin generation, prothrombin conversion and thrombin inactivation measurements in vitro can be different than in vivo. Nevertheless, thrombin generation has been shown to be a useful indicator of bleeding and thrombosis risk[2-8].

In conclusion, we show that prothrombin conversion is mainly influenced by prothrombin, FX and antithrombin levels, whereas thrombin inactivation is dependent on antithrombin and fibrinogen. Our study provides a better insight into the relation between coagulation factors and dynamic thrombin activity. The established reference values of thrombin dynamics will provide guidance values for clinically 'normal' and 'abnormal' thrombin dynamics parameter values. Our approach allows a more detailed insight into the mechanistic background of alterations of coagulation in specific patient populations and contributes in the design of therapeutic strategies in hemostatic diseases.

AUTHOR CONTRIBUTIONS

RdLK and BdL designed the project. RdLK acquired, analyzed and interpreted the data. RdLK and BdL co-wrote the manuscript. QY and MN acquired data for the manuscript, analyzed the data and critically revised it. MdM contributed crucial hemophilia samples to the project and revised the manuscript.

ADDITIONAL INFORMATION

RdLK, QY, MN, and BdL are employees of Synapse Research Institute, part of Diagnostica Stago SAS.

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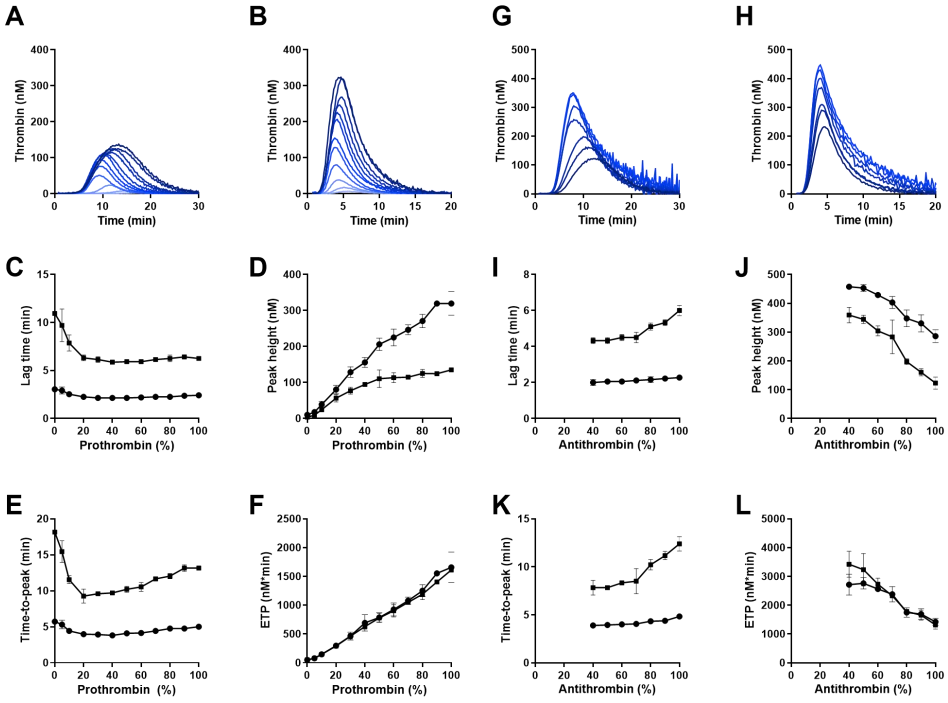
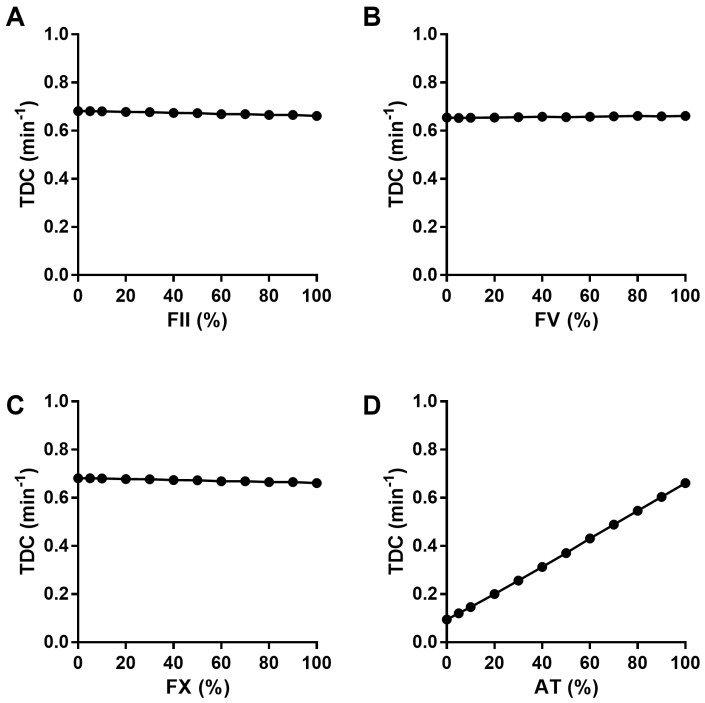
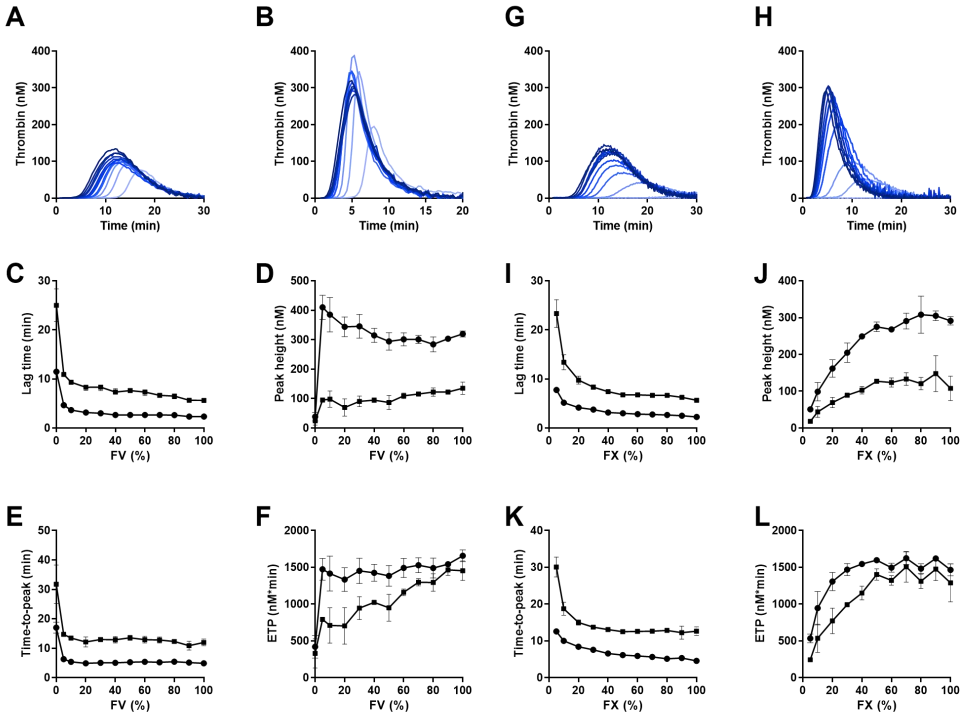
SUPPLEMENTAL MATERIAL



Supplementary figure 1: The effect of prothrombin and antithrombin on thrombin generation.(A-F) Prothrombin deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% prothrombin. Thrombin generation curves at 1 (A) and 5 pM TF (B) are shown (0-100% prothrombin from bottom to top) and thrombin generation parameters lag time (C), the peak (D), time-to-peak (E) and ETP (F) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). **(G-L)** Antithrombin deficient plasma was mixed with pooled normal to achieve plasma concentrations of 40, 50, 60, 70, 80, 90, and 100% antithrombin. Thrombin generation curves at 1 (G) and 5 pM TF (H) are shown (0-100% antithrombin from top to bottom) and thrombin generation parameters lag time (I), the peak (J), time-to-peak (K) and ETP (L) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). The average results of 3 experiments are shown as the mean ± SD.



Supplementary figure 2: The effect of coagulation factors on the thrombin decay capacity. The dose-response relationships of the thrombin decay capacity and the plasma prothrombin (A), FV (B), FX (C) and antithrombin level (D).



Supplementary figure 3: The effect of FV and FX on thrombin generation.(A-F) FV deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% FV. Thrombin generation curves at 1 (A) and 5 pM TF (B) are shown (0-100% FV from bottom to top) and thrombin generation parameters lag time (C), the peak (D), time-to-peak (E) and ETP (F) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). **(G-L)** FX deficient plasma was mixed with pooled normal to achieve plasma concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% FX. Thrombin generation curves at 1 (G) and 5 pM TF (H) are shown (0-100% FX from bottom to top and thrombin generation parameters lag time (I), the peak (J), time-to-peak (K) and ETP (L) were quantified at 1 pM TF (■ symbols) and 5 pM TF (● symbols). The average results of 3 experiments are shown as the mean ± SD.

Supplementary table 1: Reference values of thrombin dynamics parameters.

	Parameter	**Median**	**2.5th percentile**	**97.5th percentile**
1 pM TF	PC_{tot} (nM)	1036	693	1344
	PC_{max} (nM)	213	109	415
	T-AT (nM)	1001	667	1283
	T-α_2M (nM)	33	16	63
5 pM TF	PC_{tot} (nM)	1057	746	1335
	PC_{max} (nM)	250	153	474
	T-AT (nM)	1021	729	1279
	T-α_2M (nM)	34	16	63
Independent of TF concentration	TDC (min^{-1})	0.816	0.635	1.002

Supplementary table 2: Comparison of previous thrombin dynamics data with the current reference values[17-22,45].

Journal, Year	Patient population	PCtot (746-1335 nM)	PCmax (153-474 nM/min)	TAT (729-1279 nM)	Ta2M (16-63 nM)	TDC (0.633 – 1.002 min^{-1})
J Thromb Haemost. , 2015	Liver cirrhosis	Reduced	Within range	Reduced	Within range	Reduced
	Kidney failure	Reduced	Within range	Within range	Within range	Reduced
Thromb Haemost., 2016	Pediatrics	Reduced or within range	Reduced or within range	Reduced or within range	Increased or within range	Reduced or within range
Thromb Haemost., 2016	After strenuous exercise	Within range	Increased or within range	Within range	Within range	Within range
Thromb Haemost., 2016	After surgery with cardiopulmonary bypass	Reduced	Reduced or within range	Reduced	Within range or increased	Reduced
PLoS One., 2017	Severe liver cirrhosis	Reduced	Within range	Reduced	Increased in part of the patients	Reduced
Scientific Reports, 2017	Asthma patients	Within range	Data not shown	Within range	Within range	Data not shown
Blood Adv., 2018	Antiphospholipid syndrome patients	Within range or increased	Within range or increased	Data not shown	Data not shown	Within range
Thromb. Res., 2020	Severe prediatric liver disease	Reduced	Within range	Reduced	Within range or increased	Reduced or within range

Thrombin generation data obtained at 5 pM TF was analyzed; if a parameter value was outside of the normal range for a part of the patient population, this is noted in the table as either reduced or increased.

Chapter 3

Abacavir treatment in HIV patients is associated with a procoagulant thrombin generation profile

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In preparation

ABSTRACT

Background

The human immunodeficiency virus (HIV) is an enveloped retrovirus that causes HIV disease and acquired immunodeficiency syndrome (AIDS). An HIV infection can not be cured, but is treated with a combination of antiretroviral therapy (cART) that suppresses HIV replication effectively and sustainably. However, some of the therapies (e.g. abacavir) have been associated with an increased risk for thrombotic events, while others have not (e.g.Tenofovir disoproxil fumarate, TDF). The goal of our study was to compare the effect of both treatments on coagulation parameters established by thrombin generation (TG) and thrombin dynamics.

Methods

Blood was collected from 55 healthy controls and 189 HIV patients, of which 96 were treated with abacavir and 93 with TDF. TG was measured after stimulation with 5 pM tissue factor and thrombin dynamics was used to quantify prothrombin conversion and thrombin inactivation.

Results

Patients treated with abacavir and TDF had higher antithrombin levels (+3.6%, p=0.003 and +5.4%, p=0.002, respectively), resulting in a higher thrombin inactivation compared to the healthy controls (thrombin decay capacity; +6.8%, p=0.022 and +5.4%, p=0.045, respectively). In patients treated with TDF, this increase in thrombin inactivation led to a lower TG (ETP; -9.2%, p=0.003; Peak height; -10.3%, p=0.022; VelIndex; -28.1%, p=0.016) compared to healthy controls. However, as the prothrombin conversion was also higher in patients using abacavir (total prothrombin conversion; +12.5%, p=0.02), this led to a rebalanced TG profile, regardless of the lower prothrombin levels (-16.3%, p=0.001) observed in these patients. Compared to patients treated with TDF, abacavir treatment resulted in a higher TG (ETP; +6.9%, p<0.001), which again is caused by the higher prothrombin conversion (total prothrombin conversion; +9.4%, p=0.002; T-AT, +9.5%, p=0.001).

Conclusion

Treatment with abacavir was indeed associated with a higher prothrombin conversion, which led to an increase in TG. On the contrary, TDF treatment resulted in a higher thrombin inactivation and a lower TG.

INTRODUCTION

Human immunodeficiency virus (HIV) is an enveloped retrovirus that causes HIV disease and acquired immunodeficiency syndrome (AIDS)[1]. HIV enters T-helper lymphocytes, integrates into the host genome and replicates virus particles, causing a life-long infection[2]. HIV can not be cured, but the patients are treated with combination anti-retroviral therapy (cART) that can dramatically suppress the replication of the HIV-virus [3]. When cART treatment is effective, morbidity and mortality are drastically reduced [3-5]. Although these patients do not longer develop AIDS-related morbidities, more and more patients are suffering from other co-morbidities, such as cardiovascular disease (CVD) and venous thromboembolism (VTE)[3,6-13].

Patients with an HIV infection suffer from an activation of the inflammatory and haemostatic system, resulting in the elevation of, among others, C-reactive protein, interleukin 6 and D-dimers. The use of antiretroviral therapy is also associated with a persistently activated coagulation system caused by upregulation of the tissue factor pathway and a reduced anticoagulant response[14]. This was shown previously in HIV patients treated with abacavir, a nucleoside reverse-transcriptase inhibitor (NRTI), associated with an increased incidence of myocardial infarction[15-18]. Even though the underlying mechanism for these coagulation abnormalities remains unknown, it is also observed in other chronic inflammatory diseases[8,9]. Data from the SMART study showed that plasma levels of D-dimer are increased by 50% among treated HIV patients when compared to uninfected controls[19,20]. Although cART might reduce the risk of thrombotic events, in the long term cART could lead to endothelial dysfunction and vascular injury[21,22].

The coagulation system can be assessed by applying Calibrated Automated Thrombinography (CAT), which is a sensitive method to detect small alterations in coagulation. An increase of thrombin generation (TG) peak height and endogenous thrombin potential (ETP) is associated with an increased risk of thrombosis and, vice versa, a low TG peak height and ETP is associated with an increased bleeding risk[23,24]. The TG curve represents the balance between pro- and anticoagulant processes. This balance can be studied further by applying thrombin dynamics analysis that quantifies prothrombin conversion and thrombin inactivation[25,26]. In addition, thrombin dynamics analysis can be used to pinpoint changes in the pro- or anticoagulant pathway of TG induced by, for example, the use of anti-retroviral drugs[27,28].

The goal of this study was to investigate more in depth the effect of HIV treatment

with either abacavir or tenofovir disoproxil fumarate (TDF) on coagulation by determining the dynamics of TG.

METHODS

Sample collection

The population used for this study was previously described by van der Heijden et al.[29]. The study was approved by the local medical ethics committee (CMO Arnhem-Nijmegen, The Netherlands; NL425561.091.12, 2012/550). Healthy controls and cART-treated HIV patients were enrolled in the study after obtaining written informed consent. Subjects were excluded if they had either an active hepatitis B or C infection, if they had signs of other infections or if they had received coumarin derivatives or direct oral anticoagulants. Blood was collected into vacuum tubes (1 volume 0.109 mol/L trisodium citrate to 9 volumes blood; Greiner Bio-One). Platelet poor plasma (PPP) was prepared by double centrifugation at 2840 g for 10 minutes and stored at -80°C.

Thrombin generation

TG in PPP was measured using the CAT assay according to the manufacturer's standards (Diagnostica Stago, Asnière-sur-Seine, France). The conversion of the fluorescence data was done automatically by the CAT software and the following parameters were derived: lag time, time-to-peak, peak height, ETP and velocity index (VelIndex). The lag time is defined as the time point at which the burst of TG starts, which is defined as $1/6^{th}$ of the peak height. The peak height represents the highest active thrombin concentration detectable. The time-to-peak is the time until the peak height is reached. The ETP is defined as the area under the curve and represents the total thrombin potential that a plasma sample can generate. The VelIndex is the maximum slope of the propagation phase and is calculated as peak height/(time-to-peak – lag time). The generated TG curves were used in thrombin dynamics analysis, as described below.

Thrombin dynamics

The TG curve is the net result of prothrombin conversion and thrombin inactivation, and the course of prothrombin conversion can therefore be calculated from a TG curve and its thrombin inactivation[26,30]. The prothrombin conversion curve is quantified by the area

under the curve, which is defined as the total amount of prothrombin converted (PC_{tot}) during the TG test, and the peak height of the prothrombin conversion curve, which is defined as the maximum rate of prothrombin conversion (PC_{max}). The amount of thrombin-antithrombin (T-AT) and thrombin-α_2Macroglobulin (T-α_2M) complexes formed during the experiment are also quantified[26,30,31]. Additionally, the rate of thrombin inactivation was quantified by the thrombin decay constant (TDC), which is the pseudo-first order decay constant for thrombin inhibition by antithrombin, α_2M and fibrinogen.

Coagulation and inflammatory factor levels

Fibrinogen levels were measured using the Clauss method on the STart (Diagnostica Stago, France). Antithrombin levels were measured chromogenically on the automated coagulation analyzer STA-R according to manufacturer's specifications (Diagnostica Stago, Asnière-sur-Seine, France)[32]. Plasma α_2M levels were measured with an in-house chromogenic assay as previously described by Kremers et al.[26]. Prothrombin (sheep anti-human prothrombin polyclonal antibody and HRP-conjugated sheep anti-human prothrombin polyclonal antibody; Affinity Biologicals, Ancaster, Canada) levels were determined with an in-house sandwich ELISA assay[29]. Inflammatory biomarker sCD14 (Quantikine) was determined by ELISA (R&D systems, Minneapolis, USA).

Statistics

Statistical analysis was performed with SPSS version 26 and graphs were generated using Graphpad Prism software version 8. Normality of the data was assessed using the Shapiro-Wilk test. Data are presented as median with interquartile range (IQR). Comparison between groups was performed by either the Mann-Whitney test or Kruskal-Wallis test, depending on the number of groups that were compared. Fisher's exact test was used to compare nominal data between groups. The correlation matrix was performed using Spearman's correlation coefficient. P-values ≤ 0.05 were considered statistically significant.

RESULTS

TG was measured in 55 healthy controls and 189 HIV patients on stable cART treatment. The general characteristics of the healthy controls and patients are shown in Table 1. The healthy controls had a median age of 30 years (IQR, 25-52 years), and 60% were male. All HIV patients received a different cART treatment, with different combinations of drugs that included a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor (PI), an integrase inhibitor (INSTI), a nucleoside reverse transcriptase inhibitor (NRTI), a nucleotide reverse transcriptase inhibitor (NtRTI) and/or a CCR5 receptor antagonist (CCR5). From these 189 patients, 96 patients received abacavir treatment and 93 TDF treatment.

Table 1: Baseline characteristics of healthy controls and HIV patients stratified by abacavir and TDF use.

	HC	Abacavir	TDF	*Abacavir vs TDF*
n	55	96	93	
Sex (female) (%)	22 (40)	5 (5.3)	10 (10.4)	<0.001
Age (years)	30 (25-52)	50 (42-58)	53 (46-60)	0.160
BMI	23.8 (21.5-25.6)	23.8 (22.0-26.2)	24 (22-26)	0.328
Smoking, n (%)		24 (25.5)	31 (32)	0.450
HIV infection (years)		6.4 (4.2-10.3)	9.0 (6.2-15.7)	<0.001
CD4 nadir (*10^8 cells/L)		280 (185-380)	270 (135-380)	0.061
CD4 count (*10^8 cells/L)		670 (483-800)	670 (505-805)	0.375
Viral load<40 copied/mL (%)		94 (100)	96 (100)	0.658
CD4-CD8 ratio		0.79 (0.57-1.16)	0.85 (0.56-1.12)	0.886
cART duration (years)		5.3 (3.4-8.4)	7.5 (4.7-14.0)	0.002
Type of cART				
NNRTI, n (%)		16 (16.6)	41 (44.1)	<0.001
PI, n (%)		6 (6.3)	11 (11.8)	0.310
INSTI, n (%)		76 (79.2)	53 (57.0)	<0.001
NRTI, n (%)		96 (100)	93 (100)	0.99
NtRTI, n (%)		1 (1.0)	93(100)	<0.001
CCR5, n (%)		1 (1.0)	0	0.49

Note: Values are median (interquartile range) or number (percentage). Abbreviations: HC, healthy controls; TDF, tenofovir disoproxil fumarate; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; INSTI, integrase inhibitor; NRTI, nucleosidereverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; CCR5, CCR5 receptor antagonist.

Thrombin generation in HIV patients treated with abacavir or TDF

TG was measured in PPP using 5 pM tissue factor and TG parameters were quantified in healthy control subjects, HIV patients on abacavir treatment and HIV patients on TDF treatment (Figure 1). Whereas abacavir did not have a significant effect on overall TG, HIV patients on TDF treatment had a lower ETP (-9.2%, p=0.003), peak height (-10.3%, p=0.022) and velocity index (-28.1%, p=0.016) compared to healthy controls. Additionally, ETP was significantly lower in HIV patients treated with TDF than patients treated with abacavir (-6.9%, p<0.001).

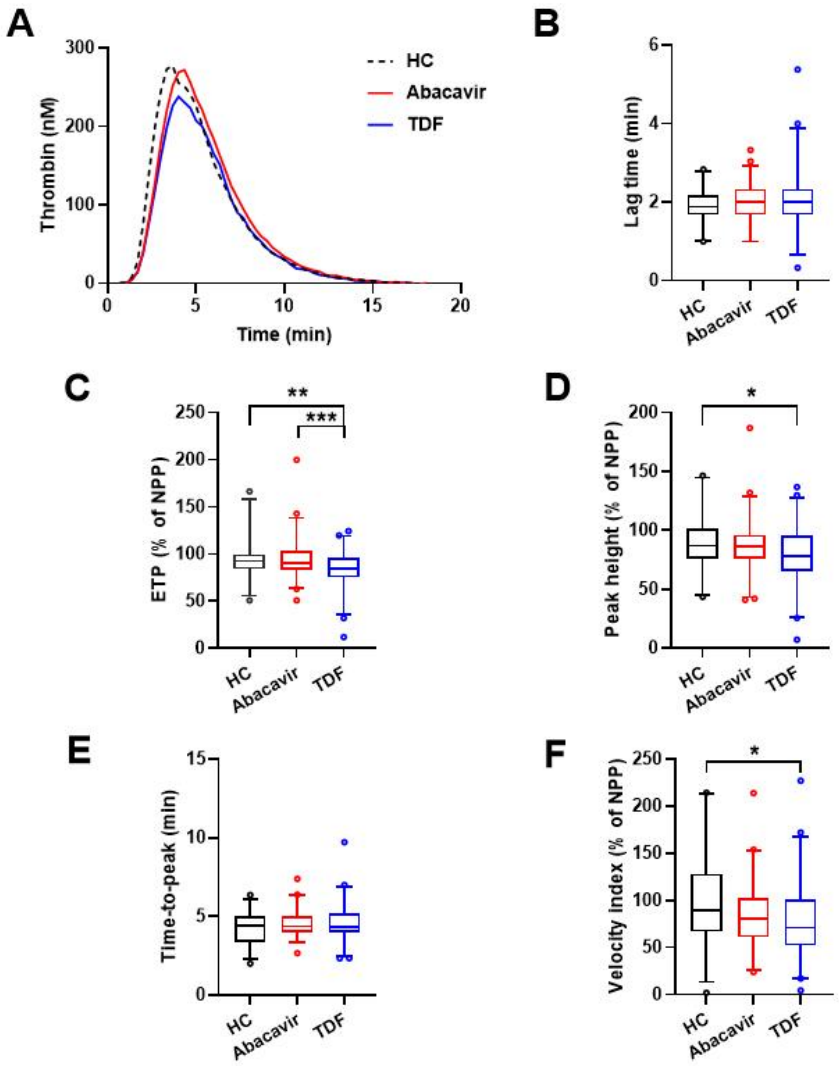


Figure 1: Thrombin generation in healthy controls and abacavir or TDF treated HIV patients. (A) Average thrombin generation curves at 5 pM TF were determined in healthy controls (black), abacavir use (red) and TDF use (blue) . TG parameters lag time (B), ETP (E), peak height (F), time-to-peak (I) and velocity index (J) were quantified. The data are shown as median and interquartile range. Statistical significance was indicated as *p<0.05, **p<0.01, ***p<0.001 using Kruskal-Wallis test.

Pro- and anticoagulant factors in HIV patients treated with abacavir or TDF

In addition to TG, we measured the plasma levels of procoagulant factors prothrombin and fibrinogen and anticoagulant factors antithrombin and α_2-macroglobulin (Table 2). HIV patients using either abacavir or TDF have significantly higher antithrombin levels (+3.6%, p=0.003 and +5.4%, p=0.002, respectively) and significantly lower prothrombin levels (-16.3%, p=0.001 and -22.2%, p<0.001, respectively) compared to healthy controls. Fibrinogen and α_2M levels did not differ between patients and healthy controls, nor between patients treated with abacavir or TDF.

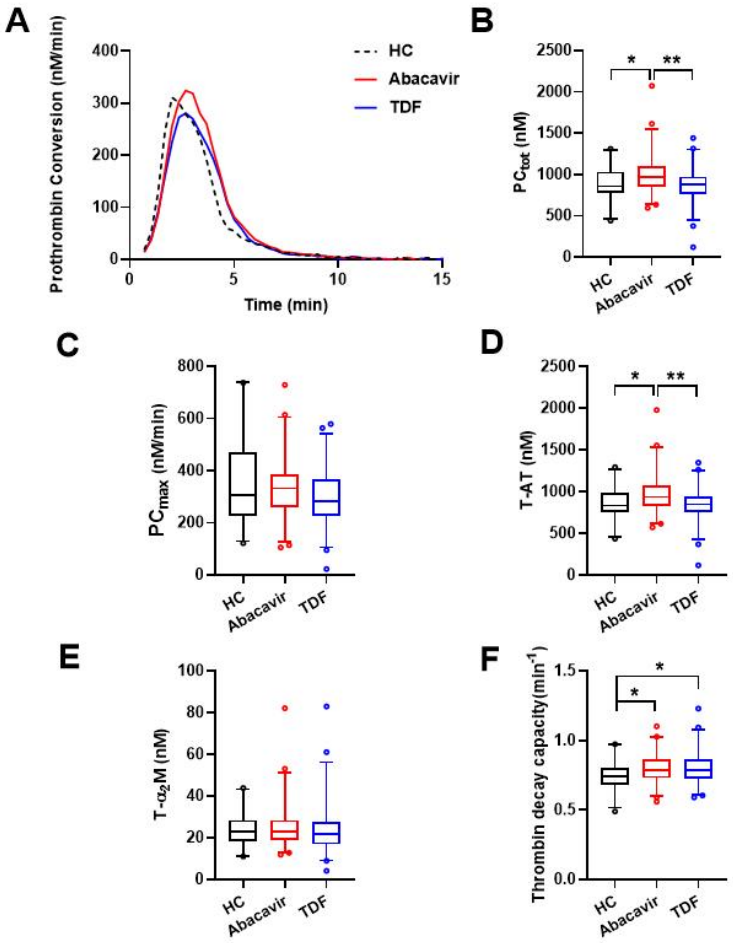


Figure 2: Thrombin dynamics in healthy controls and abacavir of TDF treated HIV patients. (C) Average prothrombin conversion curves were determined in healthy controls (black),abacavir use (red) and TDF use (blue). Total amount of prothrombin converted (PC_{tot}; D), maximal rate of prothrombin conversion (PC_{max}; G), T-AT (H), T-α_2M (K) and TDC (L) were quantified. The data are shown as median and interquartile range. Statistical significance was indicated as *p<0.05, **p<0.01 using Kruskal-Wallis test.

Thrombin dynamics in HIV patients treated with abacavir or TDF

Thrombin dynamics analysis was used to study the balance between prothrombin conversion and thrombin inactivation in more detail (Figure 3). The use of abacavir is associated with a higher prothrombin conversion (+12.5%, p=0.02), a lower TDC (+6.8%, p=0.022) and a higher T-AT complex formation (+12.0%, p=0.015). Also, treatment with TDF is associated with a significantly higher TDC (+5.4%, p=0.045), although the prothrombin conversion is comparable to the one in observed in healthy controls.

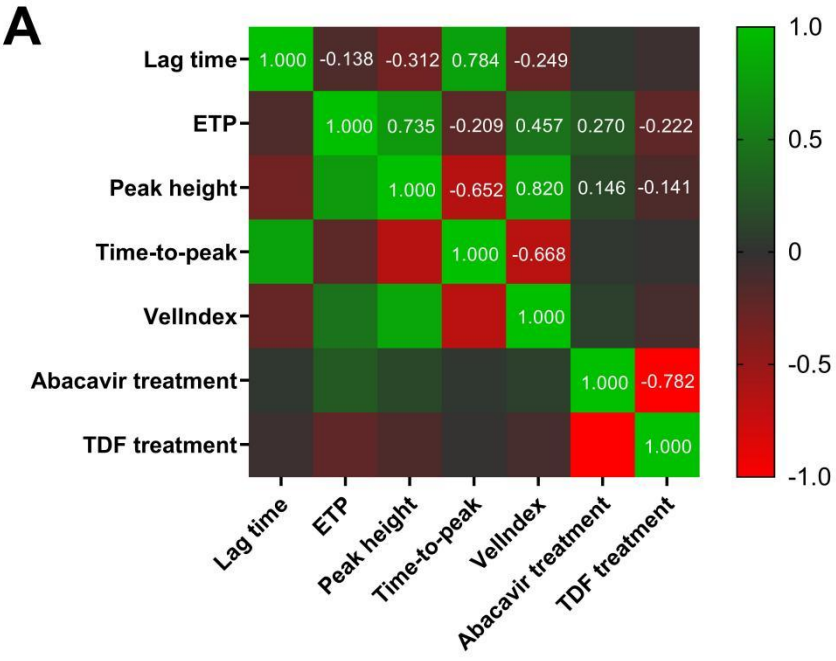
Correlation of abacavir and TDF treatment with TG and TD parameters

Figure 3 illustrates the Spearman correlation matrix for abacavir or TDF treatment with TG and TD parameters. Abacavir treatment is associated with a higher ETP, peak height, total prothrombin conversion and T-AT complex formation. In contrast, TDF use is associated with a lower ETP,peak height, prothrombin conversion and T-AT complex formation.

Table 2: Coagulation factors, thrombin generation and thrombin dynamics parameters of healthy controls and HIV patients stratified by abacavir and TDF use.

	HC (n=55)	Abacavir (n=96)	TDF (n=93)	*HC vs Abacavir*	*HC vs TDF*	*Abacavir vs TDF*
Coagulation factors						
Fibrinogen (g/L)	3.26 (2.85-4.15)	3.32 (2.77-3.98)	3.29 (2.79-4.03)	0.851	0.869	0.967
Antithrombin (%)	112 (107-119)	116 (111-123)	118 (110-123)	0.003	0.002	0.917
α_2Macroglobulin (µM)	2.21 (1.90-2.48)	1.90 (1.67-2.26)	2.11 (1.79-2.53)	0.053	0.725	0.9
Prothrombin (%)	135 (102-162)	113 (94-136)	105 (87-128)	0.001	<0.001	0.222
TG parameters						
ETP (% of NPP)	92.5 (84.0-99.5)	90.2 (82.7-102.6)	84.0 (75.0-96.0)	0.844	0.003	<0.001
Peak height (% of NPP)	87.0 (75.5-101.0)	85.8 (76.1-95.9)	78.0 (65.0-95.5)	0.385	0.022	0.084
Velocity	98.6 (63.4-124.6)	79.7 (61.3-102.5)	70.9 (52.5-99.9)	0.160	0.016	0.279
TD parameters						
PC_{tot} (nM)	859 (782-1032)	966 (856-1099)	875 (766-975)	0.020	0.912	0.002
PC_{max} (nM/min)	306 (227-471)	332 (259-387)	284 (226-367)	0.668	0.052	0.461
T-AT (nM)	835 (749-988)	935 (829-1074)	846 (750-943)	0.015	0.902	0.001
T-α_2M (nM)	23 (18-28)	23 (19-28)	22 (17-28)	0.927	0.347	0.268
TDC (min^{-1})	0.74 (0.68- 0.80)	0.79 (0.73-0.87)	0.78 (0.73-0.86)	0.022	0.045	0.800

Data is displayed as the median with between brackets the lower and higher boundary of the interquartile range. Abbreviations: PC_{tot}, total amount of prothrombin converted; PC_{max}, maximum rate of prothrombin conversion; T-AT, thrombin- antithrombin complexes; T-α_2M, thrombin-α_2-macroglobulin complexes; TDC: thrombin decay capacity


A
Lag time
ETP
Peak height
Time-to-peak
VelIndex
Abacavir treatment
TDF treatment
1.000 -0.138 -0.312 0.784 -0.249
1.000 0.735 -0.209 0.457 0.270 -0.222
1.000 -0.652 0.820 0.146 -0.141
1.000 -0.668
1.000
1.000 -0.782
1.000
1.0
0.5
0
-0.5
-1.0

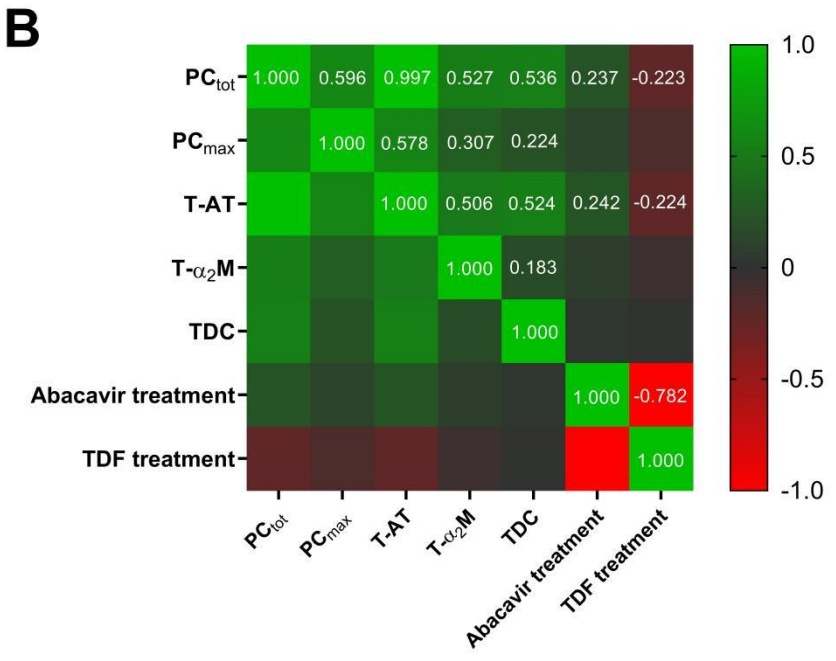

B
PCtot
PCmax
T-AT
T-α2M
TDC
Abacavir treatment
TDF treatment
1.000 0.596 0.997 0.527 0.536 0.237 -0.223
1.000 0.578 0.307 0.224
1.000 0.506 0.524 0.242 -0.224
1.000 0.183
1.000
1.000 -0.782
1.000
1.0
0.5
0
-0.5
-1.0

Figure 3. Spearman correlation matrix of coagulation capacity and cART treatment. (A) Thrombin generation parameters; (B) Thrombin dynamics parameters. Spearman's Correlation coefficients are shown when a p-value <0.05.Abbreviations: ETP, endogenous thrombin potential; VelIndex, velocity index; TDF, Tenofovir disoproxil fumarate; PC_{tot}, total amount of prothrombin converted; PC_{max}, maximum rate of prothrombin conversion; T-AT, thrombin and antithrombin complex; T-α_2M, thrombin and α_2M complex; TDC, thrombin decay capacity

DISCUSSION

We investigated the effect of abacavir and TDF use on the coagulation system in HIV-infected patients. Previously, van der Heijden et al. demonstrated a lower TG in HIV patients compared to controls, however, the authors did not explore in detail the effect of antiretroviral therapy on the coagulation system[29].

Abacavir, a guanosine analog reverse transcriptase inhibitor widely used for HIV treatment, has been associated with an increased risk of arterial thrombosis (e.g. myocardial infarction)[15,18]. TDF is a nucleotide analogue with activity against HIV that has not been associated with thrombotic complications in clinical studies[33-35]. In this study, we explored the changes in the coagulation system of abacavir and TDF treated HIV patients more in depth through TG and thrombin dynamics analysis, which quantifies the pro- and anticoagulant processes that take place during TG[36].

Interestingly, the amount of prothrombin converted during TG was increased in patients using abacavir, regardless of lower prothrombin levels. In contrast, the thrombin decay capacity (TDC), which reflects the capability of a plasma sample to inhibit thrombin, was elevated. The TDC depends on the plasma fibrinogen, antithrombin and α_2M levels[36]. We showed that patients using abacavir have higher antithrombin levels, which resulted in an elevated TDC. Subsequently, increased prothrombin conversion in combination with increased thrombin inactivation led to a new balance of TG. Moreover, abacavir use was positively associated with TG and thrombin dynamics parameters and several studies reported an association between the use of abacavir, an increased risk for arterial

thrombosis, and the development of atherosclerosis[37-41]. Therefore, higher prothrombin conversion in abacavir-treated patients revealed a pro-coagulant mechanism, which could explain the higher risk of thrombotic events in abacavir-treated patients[15,18].

In HIV patients treated with TDF, antithrombin levels are elevated to a similar extent as in abacavir patients, resulting in a similar increase of the TDC in TDF and abacavir patients. However, prothrombin conversion was unchanged in patients on TDF treatment compared to healthy subjects, resulting in a lower TG upon TDF treatment, as previously reported[42]. The lower TG and prothrombin conversion suggest a lower thrombosis risk of TDF treated patients compared to abacavir treated patients. This is in line with previous reports that HIV patients treated with TDF have a lower or similar risk of developing thrombotic events compared to the control group[33-35]. TDF is eliminated by renal instead of hepatic clearance, which reduces the risk of liver toxicity induced by drugs[43]. As most coagulation factors are produced by the liver, this could by a possible explanation for the difference in thrombotic risk associated with abacavir and TDF treatments[44-46].

In conclusion, patients treated with abacavir had an elevated prothrombin conversion and an increased thrombin inactivation, resulting in a rebalanced TG. This increase in prothrombin conversion could be one of the contributors to a more prothrombotic phenotype, as abacavir treatment has been associated with an increased risk thrombosis. In contrast, patients treated with TDF had increased thrombin inactivation but unchanged prothrombin conversion, which ultimately resulted in a lower TG profile. Therefore, TDF treated patients are expected to have a lower risk for thrombotic complications compared to HIV patients treated with abacavir.

AUTHOR CONTRIBUTIONS

QY performed experiments, analyzed the data and drafted the manuscript. RdLK performed thrombin dynamics analysis, performed analyses, supervised the data collection, and co-wrote the manuscript. MN supervised the data collection and co-wrote the manuscript. WvdH performed experiments and data analyses and revised the manuscript. LvdW

performed experiments and data analyses and revised the manuscript. HtC supervised the project and co-wrote the manuscript. BdL designed the study, supervised the project and co-wrote the manuscript. AVdV designed the study, supervised the project and co-wrote the manuscript. QdM designed the study, supervised the project and co-wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

QY, RdLK, MN and BdL are employees of Synapse Research Institute, part of Diagnostica Stago SAS.

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Chapter 4

Semi-automated thrombin dynamics applying the ST Genesia thrombin generation assay

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*Equal contribution

Submitted

ABSTRACT

Background

The haemostatic balance is an equilibrium of pro- and anticoagulant factors that work synergistically to prevent bleeding and thrombosis. As thrombin is the central enzyme in the coagulation pathway, it is desirable to measure thrombin generation (TG) in order to detect possible bleeding or thrombotic phenotypes, as well as to investigate the capacity of drugs affecting the formation of thrombin. Apart from measuring TG, by investigating the underlying processes (i.e. prothrombin conversion and thrombin inactivation), additional information is collected about the dynamics of thrombin formation.

Objectives

The objective of this study was to obtain reference values for thrombin dynamics analysis in a population of 112 healthy donors using an automated system for TG.

Methods

Blood was taken from healthy donors after obtaining informed consent. TG was measured on the ST Genesia, fibrinogen with the Clauss method on STart, antithrombin (AT) on the STA R max and α_2Macroglobulin (α_2M) levels were measured with an in-house chromogenic assay.

Results

TG was measured using the three reagent kits available for the ST Genesia: STG-BleedScreen (N=112), STG-ThromboScreen (N=112) and STG-DrugScreen (N=111). The TG data generated on the ST Genesia was used as an input for TD analysis, in combination with the plasma levels of AT, α_2M and fibrinogen that were 113% (108% - 118%), 2.6 µM (2.2 µM – 3.1 µM) and 2.9 g/L (2.6 g/L – 3.2 g/L), respectively. PC_{tot} and PC_{max} increased with increasing TF concentration. PC_{tot} increased from 902 nM to 988 nM, whereas PC_{max} from 172 nM/min to 508 nM/min. Thrombin (T)-AT and T-α_2M complexes also increased with increasing TF concentration (i.e. from 860 nM to 955 nM and from 28 nM to 33 nm, respectively). PC_{tot}, T-AT and T-α_2M complex formation were strongly inhibited by the addition of TM (-44%, -43% and -48%, respectively), whereas PCmax was

affected less (-24%). PC_{tot}, PC_{max}, T-AT and T-α_2M were higher in women using OC compared to men and women without OC and inhibition by TM was also significantly less in women on OC ($p<0.05$).

Conclusions

We showed that TG measured on the ST Genesia device can be used as an input for thrombin dynamics analysis. The data obtained can be used as reference values for future clinical studies as the balance between prothrombin conversion and thrombin inactivation has shown to be useful in several clinical settings.

INTRODUCTION

The haemostatic balance is an equilibrium of pro- and anticoagulant factors that work synergistically to maintain haemostasis and prevent bleeding and thrombosis. When coagulation is triggered, both intrinsic and extrinsic coagulation pathways come together in one common pathway that finally leads to the development of thrombin [1]. Thrombin is the key player of the coagulation cascade as it not only cleaves fibrinogen into fibrin monomers, but it also evokes the activation of coagulation factors (F) V, VIII and XI, as well as the feedback activation of anti-coagulant proteins (e.g. antithrombin, thrombomodulin (TM) and tissue factor (TF) pathway inhibitor [2].

As thrombin has various roles in the regulation of the coagulation cascade, it is desirable to measure thrombin generation (TG) in order to detect possible bleeding or thrombotic phenotypes. The semi-automated Calibrated Automated Thrombinography (CAT) method has been shown to be associated with the risk of bleeding and thrombosis in clinical studies [3-9]. It is a sensitive tool that identifies congenital or acquired haemostatic disorders, and can be used to investigate the efficacy of drugs that affect the formation of thrombin. Nowadays, the ST Genesia performs a fully automated TG measurement in platelet poor plasma [10-12].

Several years ago, the add-on thrombin dynamics (TD) analysis was developed for the CAT method [13]. This computational method allows the researcher to study the pro- and anticoagulant processes that determine TG: prothrombin conversion and thrombin inactivation. Over the past years, the TD method has shown its usefulness in multiple clinical settings [14-22]. TD analysis can pinpoint changes in prothrombin conversion or thrombin inactivation that provoke a change in coagulation. In liver cirrhosis patients, TD analysis provided evidence that indeed TG is rebalanced and that the prothrombin conversion and thrombin inactivation are both reduced due to lower coagulation factor production [18]. Moreover, as the TD method partially relies on the computational modeling of the inactivation of thrombin, *in silico* experimentation can be used to predict the effect of changes in prothrombin conversion and thrombin inactivation on the TG curve. *In silico*

modelling was used in several clinical settings, for example to study the effect of prothrombin complex concentrate administration with and without added antithrombin after cardiopulmonary bypass surgery [16] and to prediction of the effect of antithrombin expression targeting in haemophilia patients and investigate the inter-patients variability [23].

As TD analysis has been shown to be clinically relevant in a research setting, we set out to perform TD analysis using TG curves acquired on the ST Genesia, which is available in clinical laboratories. We determined reference values for all ST Genesia acquired TD parameters using all currently available ST Genesia reagents (STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen) in 112 healthy donors. Moreover, we investigated the effect of sex and oral contraceptive (OC) on TD parameters.

MATERIALS AND METHODS

Blood collection

The population described in this study was previously investigated in a study by Ninivaggi et al. [10]. In total, 112 healthy donors were included in this study. The study was conducted according to the Declaration of Helsinki (2013) and approved by the Medical Ethical Committee of the Maastricht University Medical Centre. Non-inclusion criteria for this study were the use of drugs interfering with coagulation, having a known coagulation disorders and/or being younger than 18 or older than 65 years. Blood samples were taken from the donors only after signing the informed consent. Vacuum blood drawing tubes (Greiner Bio-One) containing 3.2% sodium citrate (in a 9:1 ratio blood:citrate) were used to draw the blood from the antecubital vein. Platelet poor plasma was obtained by centrifuging the blood twice for ten minutes at 2630g at room temperature immediately after blood drawing. The anonymized plasma samples were stored at -80°C until further use.

Thrombin generation

The ST Genesia was used to measure TG as previously described [10]. In short, first a calibration curve was performed by using the STG-Cal&Fluo kit. Once the calibration has

been performed successfully, the reference plasma and quality controls were measured. For this study three reagent kits were used that are commercially available: STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen. Each reagent kit contains its own reference plasma that is used to normalize the sample thrombin generation data, as well as 2 or 3 quality controls. The STG-ThromboScreen reagent kit contains two activators with the same TF and phospholipid content, but one of them also contains rabbit-derived TM. After successful completion of these runs, the plasma samples of the healthy donors were thawed in a warm water bath at 37°C for 5 minutes. Immediately hereafter, the plasma tubes were gently mixed, placed on board and TG was measured. The samples were run in duplicate and the ST Genesia embedded software analyzed the data automatically and gave a mean result for the duplicates. The reference plasma run in parallel to the samples, comes with specific assigned ranges provided on a barcoded flyer and helps to normalize results [24,25]. The ST Genesia normalizes the results for each sample automatically by applying the flowing formula: [patient sample result / reference plasma result · activity assigned for the particular lot and parameter of this reference plasma].

Plasma levels of antithrombin, fibrinogen and α_2Macroglobulin

Antithrombin, fibrinogen and α_2Macroglobulin (α_2M) levels were measured to perform TD analysis. Antithrombin was measured on the automated coagulation analyzer STA-R Max according to manufacturer specifications (Diagnostica Stago, Asnières-sur-Seine, France) using STAR-Stachrom ATIII kit. Functional fibrinogen levels were measured using the Clauss method with STAR-Liquid Fib on STart (reagent and semi-automated analyzer Diagnostica Stago, Asnières-sur-Seine, France). Plasma α_2M levels were measured with an in-house chromogenic assay as previously described by Kremers et al. [13].

Thrombin dynamics

The TG curve is the net result of prothrombin conversion and thrombin inactivation [13]. The course of prothrombin conversion can be calculated if TG and thrombin inactivation are

known. Thrombin inactivation was determined by the previously described computational model which is based on the plasma concentrations of antithrombin, α_2M and fibrinogen [13], and used to determine prothrombin conversion curves from TG data [22]. The total inhibitory potential of each plasma was quantified by the thrombin decay constant (TDC). Additionally, four parameters were quantified from the prothrombin conversion curves: PC_{tot} (total amount of prothrombin converted during the thrombin generation test), PC_{max} (maximum rate of prothrombin conversion), T-AT (amount of thrombin-antithrombin complexes formed) and T-α_2M (amount of thrombin-α_2M complexes formed) [13,22].

Statistics

The GraphPad Prism software (version 8.4.2, San Diego, CA) was used to determine statistical significance of the results. Data are presented as median ± interquartile range (IQR) as indicated. Data were checked for normality by the Shapiro-Wilk test. As not all groups passed normality, the non-parametrical Dunn's multiple comparisons test was used for statistical comparison. The Friedman test was used when comparing paired results. Reference ranges were established by calculating the 2.5th and 97.5th percentile in the dataset of 112 healthy subjects. A p-value <0.05 was considered statistically significant.

RESULTS

Thrombin generation measured on the ST Genesia

TG was measured using the three reagent kits available for the ST Genesia: STG-BleedScreen (N=112), STG-ThromboScreen (N=112) and STG-DrugScreen (N=111). The TF concentrations of the aforementioned reagent kits are manufacturer proprietary information, however, they range from low to medium and high, respectively. Table 1 summarizes the following TG parameters: lagtime, peak height, time-to-peak, ETP, velocity index and ETP inhibition for all reagent kits. As expected, peak height and velocity index increased with increasing TF concentration, while lagtime and time-to-peak shorted with increasing TF concentration. The effect on ETP was less pronounced compared to the other

parameters. Addition of TM affected especially peak height and ETP, and to a minor extent velocity index, lagtime and time-to-peak. The median (IQR) ETP inhibition was 47% (34.9% – 64.3%).

Table 1: Thrombin generation data. TG was measured with the ST Genesia using the STG-BleedScreen (N=112), STG-DrugScreen (N=111) and STG-ThromboScreen (N=112). Data are median with IQR. Abbreviations: ETP, endogenous thrombin potential; TM, thrombomodulin; IQR, interquartile range.

	STG-BleedScreen		STG-ThromboScreen		STG-DrugScreen	
	Median	IQR	Median	IQR	Median	IQR
Lagtime (min)	2.9	2.6 - 3.4	2.3	2.1 - 2.6	1.3	1.2 - 1.5
Peak (nM)	168	140 - 204	195	169 - 238	347	300 - 405
Time-to-peak (min)	6.4	5.8 - 7.3	5.4	4.6 - 6.2	2.8	2.5 - 3.3
ETP (nM*min)	1112	934 - 1325	1127	993 - 1388	1213	1087 - 1419
Velocity Index (nM/min)	60	45 - 81	84	63 - 115	328	231 - 417

Thrombin dynamics on ST Genesia TG data

The TG data generated on the ST Genesia was used as an input for TD analysis, in combination with the plasma levels of antithrombin, α_2M and fibrinogen that were measured for each subject. The median values (IQR) for AT, α_2M and fibrinogen were 113% (108% - 118%), 2.6 µM (2.2 µM – 3.1 µM) and 2.9 g/L (2.6 g/L – 3.2 g/L), respectively.

The TD parameters PC_{tot}, PC_{max}, T-AT and T-α_2M are shown in figure 1. PC_{tot}, increased slightly with increasing TF concentration from 902 nM to 933 nM to 988 nM with the STG BleedScreen, STG ThromboScreen and STG DrugScreen, respectively. On the contrary, PC_{max} increased strongly with increasing TF concentration and ranged from 172 nM/min to 206 nM/min to 508 nM/min for the aforementioned reagent kits. Similarly to the ETP, T-AT and T-α_2M complexes increased with the TF concentration. T-AT was, respectively for the STG BleedScreen, STG ThromboScreen and STG DrugScreen, 860 nM,

897 nM and 955 nM, while T-α_2M was 28 nM, 30 nM and 33 nM. The effect of TM was measured using the STG-ThromboScreen kit, which as previously described contains TG trigger with and without TM. PC_{tot}, T-AT and T-α_2M complex formation are strongly inhibited by the addition of TM (-44%, -43% and -48%, respectively, Figure 1B, F and H), whereas PC_{max} was affected less, but still significantly (-24%, Figure 1 D).

Effect of sex and oral contraceptive use

It has been previously reported that TG and TD parameters [22] differ between men and women, and that the use of OC can influence TG in women [10,12,26]. Here, we compared three groups: men, women without the use of OC and women taking OC. No significant differences in TG parameters between men and women without OC were found, except for the lagtime, that was consistently shorter in women (Supplementary table 1). On the contrary, women taking OC showed not only a shorter lag time and time-to-peak, but also a higher peak height and ETP, compared to women that did not use OC or men. A more pronounced consequence of OC usage could be observed after addition of TM. Addition of TM affected ETP inhibition to a lesser extent in women using OC as the median ETP inhibition was only 25.5% (22.3% – 32.3%) compared to 57.6% (43.4% - 65.8%; $p<0.0001$) and 45.0% (34.9% - 63.3%; $p=0.0006$) in men and women without OC, respectively.

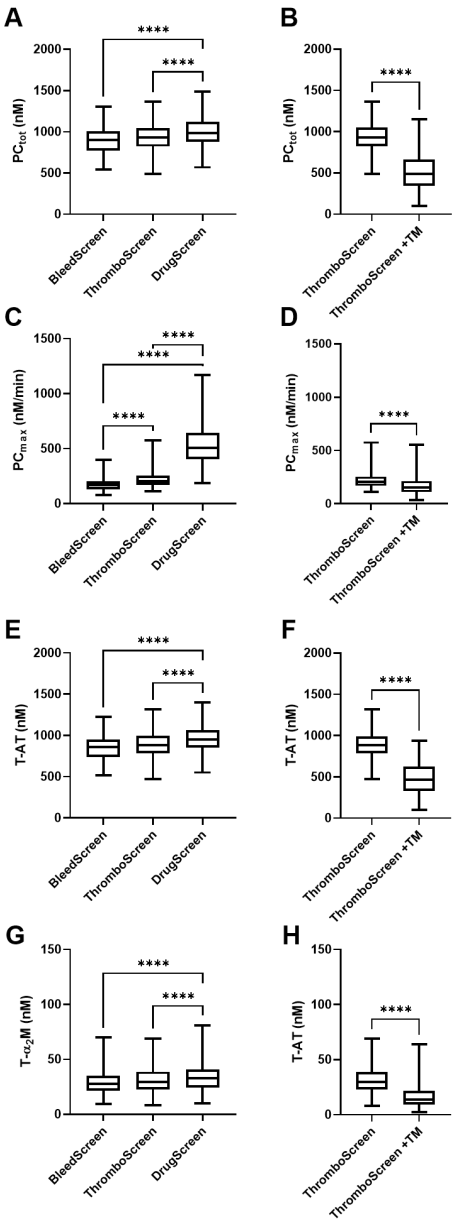

Figure 1: Thrombin dynamics parameters obtained from thrombin generation data measured with the ST Genesia. TD analysis was performed on the TG data measured with the STG-BleedScreen (N=112), STG-DrugScreen (N=111) and STG-ThromboScreen with and without TM (N=112). TD parameters PCtot (A and B), PCmax (C and D), T-AT (E and F) and T-α_2M (G and

H) complexes are depicted. Data are median with interquartile ranges and minimum and maximum values. Abbreviations: tot, total; max, maximum; TM, thrombomodulin; T-AT, thrombin-antithrombin complex; T-α_2M, thrombin-α_2M complex.

The TD parameters were compared between men, women without OC and with OC for all ST Genesia reagents (Figure 2). PC_{tot} did not differ between men and women without OC, regardless of the reagent used. However, PC_{tot} was significantly higher in women using OC compared to women without OC if TG was measured with the STG-BleedScreen reagent (+21 %; p=0.017), and in the presence of TM (STG-ThromboScreen+TM reagent; +64 %; p<0.001). T-AT complexes followed the same trend as PC_{tot}: no difference was observed between men and women, but women on OC showed a higher T-AT formation than women without OC, for the STG-BleedScreen (948 nM vs 784 nM; p=0.009) and STG-ThromboScreen+TM (773 nM vs 482 nM; p=0.001) reagents.

PC_{max} was comparable between men and women without OC, but significantly higher in women using OC than in men, regardless of the trigger used (+49% up to +158% depending on the trigger reagent, all p<0.001). Also, compared to women without OC, PC_{max} was significantly higher (+41% up to +127% depending on the trigger reagent, all p<0.001). T-α_2M formation was significantly higher in women using OC than men for the STG-BleedScreen (+52%, p=0.04), STG-ThromboScreen (+39%, p=0.04), and STG-ThromboScreen+TM (+133%, p<0.001). Furthermore, in the presence of TM, T-α_2M formation was significantly higher in women without OC compared to men (+33%, p=0.04), and in women using OC compared to women without OC (+75%, p=0.01).

We investigated further whether differences AT, α_2M and fibrinogen could explain the differences in TD parameters in men and women and in association with OC use (Figure 3). Plasma antithrombin levels were significantly higher in men than in women without and with OC (p=0.0148 and p=0.001, respectively). α_2M and fibrinogen were significantly lower in men compared to women without OC (p=0.0146 and p=0.0472, respectively). OC use did not cause any significant difference in plasma AT levels, α_2M or fibrinogen.

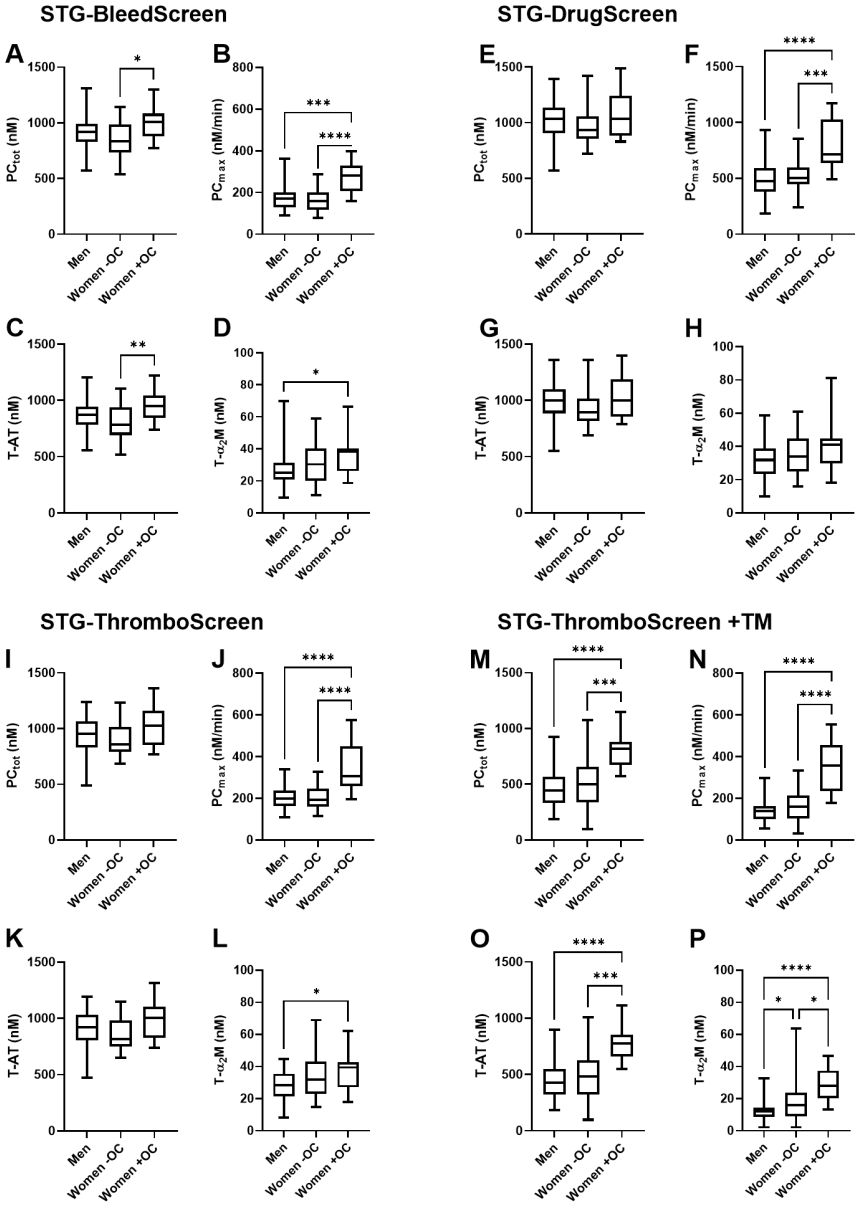

Figure 2: Thrombin dynamics parameters obtained from thrombin generation measured with the ST Genesia stratified for sex and oral contraceptive use. TG was measured with the STG-BleedScreen (panel A-D); STG-DrugScreen (panel E-H), STG-ThromboScreen without TM

(panel I-L) and STG-ThromboScreen with TM (panel M-P). TD parameters PC_{tot} (panels A, E, I and M), PC_{max} (panels B, F, J and N), T-AT (panels C, G, K and O) and T-α_2M (panels D, H, L and P) complexes are depicted for each group: men, women without OC and women with OC. Data are median with IQR and minimum and maximum values. Abbreviations: tot, total; max, maximum; T-AT, thrombin-antithrombin complex; T-α_2M, thrombin-α_2M complex; TM, thrombomodulin.

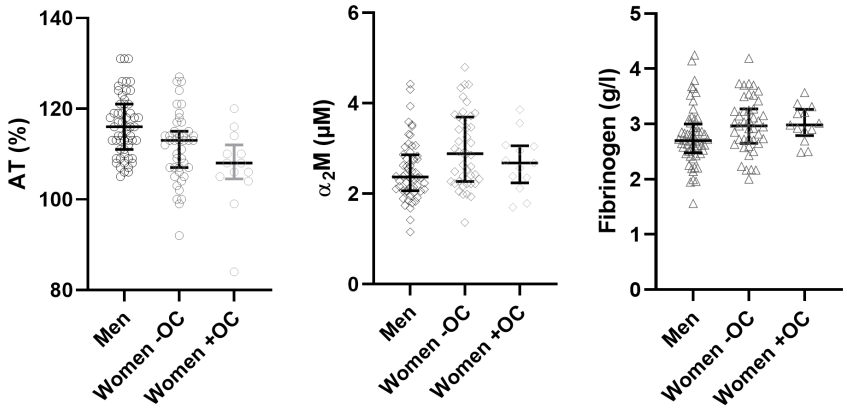

Figure 3: Antithrombin, α_2M and fibrinogen levels. The coagulation factor levels were measured in the plasma of 112 healthy donors and are depicted for the three groups: men, women without OC and women with OC. Data are median with IQR. The grey part indicates the median of the total population (interrupted line) with the IQR (dotted line). Abbreviations: AT, antithrombin; OC, oral contraceptives.

Reference values for thrombin dynamics parameters on ST Genesia

Reference values for each TD parameter and STG reagent were calculated as the 2.5th and 97.5th percentile of the distribution using the data from all 112 healthy donors (Table 2). The reference range for PC_{tot} was 609-1239 nM, 654-1416 nM, 542-1251 nM and 188-1001 nM for STG-BleedScreen, STG-DrugScreen, STG-ThromboScreen and STG-ThromboScreen+TM reagents, respectively. For PC_{max}, the reference ranges were 90-364 nM/min, 232-1088 nM/min, 115-472 nM/min and 56-466 nM/min, for

STG-BleedScreen, STG-DrugScreen, STG-ThromboScreen and STG-ThromboScreen+TM reagents. Respectively for STG-BleedScreen, STG-DrugScreen, STG-ThromboScreen and STG-ThromboScreen+TM reagents, T-AT reference ranges were: 586-1182 nM, 630-1364 nM, 526-1202 nM, and 184-870 nM. For T-α_2M, respective reference ranges were 12-60 nM for STG-BleedScreen, 17-59 nM for STG-DrugScreen, 15-56 nM for STG-ThromboScreen, and 4-41 nM for STG-ThromboScreen+TM.

Table 2: Reference ranges for thrombin dynamics parameters determined on the ST Genesia. TG was measured with STG-BleedScreen, STG-DrugScreen and STG-ThromboScreen on the ST Genesia in 112 healthy subjects and reference ranges were calculated as the 2.5th and 97.5th percentile of the distribution.

	Median	2.5th percentile	97.5th percentile
STG-BleedScreen			
PC_{tot} (nM)	902	609	1239
PC_{max} (nM/min)	172	90	364
TAT (nM)	860	586	1182
Ta_2M (nM)	28	12	60
STG-DrugScreen			
PC_{tot} (nM)	988	654	1416
PC_{max} (nM/min)	508	232	1088
TAT (nM)	955	630	1364
Ta_2M (nM)	33	17	59
STG-ThromboScreen			
PC_{tot} (nM)	933	542	1251
PC_{max} (nM/min)	206	115	472
TAT (nM)	897	526	1202
Ta_2M (nM)	30	15	56
STG-ThromboScreen +TM			
PC_{tot} (nM)	492	188	1001
PC_{max} (nM/min)	153	56	466
TAT (nM)	470	184	870
Ta_2M (nM)	14	4	41

DISCUSSION

TD analysis pinpoints changes in the hemostatic balance to the pro- or anticoagulant pathway during thrombin generation [13,22]. Several studies using TD analysis based on TG data generated by the semi-automated CAT method have shown its clinical relevance [14,16-21,27-30]. We set out to perform TD analysis using TG curves acquired on the ST Genesia, which is currently available in clinical laboratories. We determined the reference values for all TD parameters using the currently available ST Genesia reagents (STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen) in a population of 112 healthy donors. Moreover, we investigated the effect of sex and the use of oral contraceptive (OC) on TD parameters.

An additional advantage of TD analysis is that it offers the possibility to perform *in silico* modelling of the changes in TG when plasma coagulation factor levels change. For example, in the past TD analysispointed out that the choice of prothrombin complex concentrates containing antithrombin are probably safer for treatment of bleeding in liver cirrhosis patients than prothrombin complex concentrates without antithrombin [18]. Indeed, this was confirmed by a clinical study performed by the group of Lisman et al., who found that although treatment with prothrombin complex concentrates without antithrombin increased TG by 2-4 fold, whereas the administration of fresh frozen plasma, containing both pro- and anticoagulants, increased TG only slightly [31]. Additionally, the *in silico* prediction of the increase of TG by silencing miRNA targeting antithrombin expression in individual hemophilia patients is expected to improve the dose targeting of the drug [22,23].

Since the measurement of TG has now been fully automatized in the ST Genesia analyzer, TG curves can now also be acquired in clinical laboratories [32-37]. Therefore, we investigated the use of TD analysis using ST Genesia TG data to establish normal ranges for TD parameters in an apparently healthy population. The reference values for TD parameters obtained from ST Genesia TG curves are very comparable to the referenced values previously determined for TD parameters obtained from CAT data [22]. TD parameters determined with the STG-BleedScreen reagent, which contains the lowest amount of TF,

corresponds well to TD parameters measured with the PPP Reagent Low used in the CAT method [22]. Similarly, TD parameters measured with the STG-ThromboScreen reagent without TM, containing an intermediate level TF concentration, corresponded well to TD parameter values obtained with the CAT PPP Reagent, containing an intermediate TF dose [22].

We also investigated the effect of sex and OC use on the TD parameters, as these are known to affect TG [10,38] and coagulation tests in general [39,40]. In this study we demonstrated that TD parameters did not differ between men and women without OC. The only difference found in TD parameters between men and women is the higher amount of T-a_2M complexes found in women compared to men. In CAT TD we found similar results, including a (non-significant) trend towards higher T-α_2M formation in women [22]. This discrepancy between men and women can be attributed to the higher level of α_2M in women compared to men, which stimulates the inhibition of thrombin by α_2M [22,41,42].

On the contrary, the use of OC has distinctive effects on TD parameters, including the attenuation of the inhibitory effect of TM, as known from literature [43,44]. The use of OC causes a significant increase of almost all TD parameters, both in ST Genesia- and CAT-based TD analysis, in the absence of TM [22]. The use of OC increases TG through the stimulation of both the total amount and the maximal rate of prothrombin conversion (PC_{tot} and PC_{max}). Although this effect is more pronounced in the presence of TM, it was also observed in its absence. We have previously shown that plasma prothrombin and FX levels are important influencers of prothrombin conversion, as both PC_{tot} and PC_{max} increase dose-dependently with the prothrombin and FX level [22]. Remarkably, the effect of FV levels on PC_{tot} and PC_{max} was marginal around its physiological plasma concentration, indicating that OC use might have additional effects on prothrombin conversion besides the well known inhibitory effect on the activated protein C pathway [38-40]. This could also explain why the effect is also detected in the absence of TM.

In conclusion, we showed that TG curves measured on the fully automated ST Genesia device can be used as an input for TD analysis. The data obtained can be used as

reference values for future clinical studies as the balance between prothrombin conversion and thrombin inactivation has shown to be useful in several clinical settings. Therefore, the introduction of the ST Genesia to the clinic is a future opportunity to also use the TD method in specified clinical settings.

AUTHORSHIP DETAILS

AC and BdL designed the study. RdLK collected the samples. MN and QY performed the experiments. MN and RdLK analyzed the data and wrote the first draft of the manuscript. AC and BdL critically revised the manuscript.

CONFLICT OF INTEREST

A. Carlo is full-time employee of Diagnostica Stago SAS. Q. Yan, M. Ninivaggi, R. de Laat-Kremers and B. de Laat are employees of Synapse Research Institute, part of Diagnostica Stago S.A.S..

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: Thrombin generation in men and women with and without the use of oral contraceptives. TG was measured with STG-BleedScreen, STG-DrugScreen and STG-ThromboScreen on the ST Genesia. Data shown are median with IQR (N=112). Abbreviations: IQR, interquartile ranges; OC, oral contraceptives; M, men; W-, women without OC; W+, women with OC; ETP, endogenous thrombin potential; Vel. Index, velocity index; TM, thrombomodulin.

	Men (N=56)		Women -OC (N=43)		Women +OC (N=13)		p-value		
	Median	IQR	Median	IQR	Median	IQR	M/W-	M/W+	W-/W+
STG-BleedScreen									
Lagtime (min)	3.1	2.8 - 3.6	2.7	2.5 - 3.2	2.5	2.1 - 2.7	0.0042	<0.0001	n.s.
Peak Height (nM)	162	140 - 198	164	120 - 194	271	204 - 311	n.s.	<0.0001	<0.0001
Time-to-peak (min)	6.7	6.1 - 7.6	6.3	5.8 - 7.3	5.2	4.4 - 6.0	n.s.	0.0002	0.0146
ETP (nM.min)	1092	922 - 1236	1064	900 - 1320	1374	1141 - 1534	n.s.	0.0036	0.0031
Vel. Index (nM/min)	59	41 - 75	60	43 - 78	131	77 - 170	n.s.	<0.0001	0.0001
STG-DrugScreen									
Lagtime (min)	1.4	1.3 - 1.6	1.3	1.2 - 1.4	1.2	1.1 - 1.3	0.0110	0.0010	n.s.
Peak Height (nM)	334	294 - 376	335	301 - 405	483	392 - 516	n.s.	<0.0001	0.0010
Time-to-peak (min)	3.1	2.6 - 3.5	2.7	2.4 - 3.0	2.5	2.1 - 2.5	0.0125	0.0001	n.s.
ETP (nM.min)	1185	1088 - 1405	1223	1057 - 1396	1409	1151 - 1804	n.s.	n.s.	0.0487
Vel. Index (nM/min)	299	199 - 365	337	276 - 438	512	406 - 821	n.s.	<0.0001	0.0009
STG-ThromboScreen									
Lagtime (min)	2.5	2.2 - 2.8	2.2	2.0 - 2.6	2.0	1.8 - 2.2	0.0235	0.0006	n.s.
Peak Height (nM)	190	169 - 217	193	160 - 230	280	239 - 374	n.s.	<0.0001	0.0001
Time-to-peak (min)	5.7	5.1 - 6.5	5.1	4.7 - 6.2	4.4	3.6 - 4.8	n.s.	<0.0001	0.0154
ETP (nM.min)	1130	967 - 1368	1100	968 - 1310	1414	1140 - 1738	n.s.	0.0132	0.0132
Vel. Index (nM/min)	72	59 - 98	83	63 - 112	160	115 - 263	n.s.	<0.0001	0.0001
STG-ThromboScreen +TM									
Lagtime (min)	2.5	2.2 - 2.8	2.1	2.0 - 2.5	2.0	1.8 - 2.1	0.0252	0.0002	n.s.
Peak Height (nM)	114	81 - 136	136	91 - 181	303	196 - 328	n.s.	<0.0001	<0.0001
Time-to-peak (min)	4.7	4.3 - 5.1	4.4	4.1 - 4.8	3.9	3.4 - 4.3	n.s.	0.0004	0.0345
ETP (nM.min)	483	368 - 658	601	425 - 803	1100	871 - 1262	n.s.	<0.0001	<0.0001
Vel. Index (nM/min)	65	45 - 79	80	50 - 118	202	122 - 296	n.s.	<0.0001	0.0001
ETP inhibition (%)	57.6	43.4 - 65.8	45.0	34.9 -63.3	25.5	22.3 - 32.2	n.s.	<0.0001	0.0006

Chapter 5

Reference values for thrombin dynamics in platelet rich plasma

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ABSTRACT

Thrombin generation (TG) is a better determinant of the overall function of the hemostatic system than routinely used clotting time-based assays and can be studied more in detail by thrombin dynamics analysis. Platelet poor plasma is often used to measure TG, however, measuring the contribution of the platelets is also important as patients with a low platelet count or with dysfunctional platelets have an increased risk of developing bleeding. In this study, platelet rich plasma (PRP) was collected from 117 healthy individuals. PRP was measured undiluted and diluted to a varying platelet concentration of 10 x 10^9/L to 400 x 10^9/L. Prothrombin conversion and thrombin inactivation were calculated from the data obtained by the TG parameters and coagulation factor levels (antithrombin, α_2Macroglobulin (α_2M) and fibrinogen). Reference ranges of TG and thrombin dynamics in PRP of 117 healthy individuals were established. Peak, velocity index and the maximum rate of prothrombin conversion increased linearly with platelet count, but endogenous thrombin potential reached a maximum at 150 x 10^9/L as seen in a subset population (n=20). More extensive analysis revealed that a platelet count below 50 x 10^9/L did not affect TG parameters (except for the ETP). Correlation analysis indicated that the platelet count mainly affected the rate of prothrombin conversion. Inhibition of thrombin by antithrombin and α_2M increased with increasing TG, but the ratio of inhibition by antithrombin or α_2M remained the same independently of the total thrombin formed. In conclusion, TG and thrombin dynamics were assessed in PRP of healthy donors to provide reference values for future TG studies in PRP. Increasing the platelet count mainly affected the rate of prothrombin conversion and TG, rather than the total amount of thrombin formed.

INTRODUCTION

It is well-established that measuring thrombin generation (TG) is a better determinant of the overall function of the hemostatic system than routinely used clotting time-based assays (e.g. prothrombin time and activated Partial Thromboplastin Time) [1-5]. The Calibrated Automated Thrombogram (CAT) assay was designed to measure TG in platelet poor (PPP) and rich plasma (PRP), as well as in whole blood after some adaptions [6]. Measuring TG is a very sensitive method to distinguish normal conditions from hypo- and hypercoagulable states [6-8], as well as to investigate the contribution of natural pro- and anticoagulants and the effect of drugs affecting coagulation [6,9,10]. Nevertheless, it is not possible to study all mechanistic changes related to the haemostatic coagulation system in detail by measuring TG alone. Kremers et al. developed a computational approach to calculate parameter values for both pro- and anticoagulant mechanisms (prothrombin conversion and thrombin inactivation, respectively) during TG. The computational model takes into account the measured TG, as well as the plasma levels of fibrinogen, that plays a modulating role, and the thrombin inhibitor levels of α_2Macroglobulin (α_2M) and antithrombin [11-14]. By quantifying prothrombin conversion and thrombin inactivation capacities, more information is gained about the pro- and anticoagulant processes that underlie TG [11,15,16].

Although the clinical perspective needs to be further explored, measuring TG and thrombin dynamics in PRP can contribute to a better understanding of the involvement of platelets, which are crucial for coagulation *in vivo* [1,2,17,18]. Especially for patients with a platelet disorder or for patients taking drugs that affect platelet function, it is interesting to investigate the overall thrombin potential in PRP. As the platelet count is important for TG, investigating the effect of a varying platelet count can elucidate the kinetics of TG in patients with platelet deficiencies, as e.g. in thrombocytopenia [19-23].

The goal of this study was to establish the reference ranges of TG and thrombin dynamics in PRP of 117 healthy donors, as well as to determine the effect of platelet count on the thrombin dynamics in these healthy donors.

METHODS

Blood collection

Blood samples were taken from 117 healthy donors that were not taking any medication interfering with coagulation. Before blood drawing, all donors gave their written informed consent. The study was approved by the Medical Ethical Committee of the Maastricht

University Medical Centre and conducted according to the Declaration of Helsinki (2013). Blood (9 volumes) was aseptically drawn in vacutainer tubes (Greiner Bio-One) containing 3.2% sodium citrate (1 volume), from the antecubital vein of healthy subjects. The blood was kept at room temperature (± 21°C) and used within 2 hours.

Preparation of platelet poor and rich plasma

PRP was prepared by centrifuging the blood for 15 minutes at 250 g without a brake. As we were interested in investigating the effect of platelet count on TG and thrombin dynamics, the PRP was diluted with autologous PPP to the indicated platelet count. The PPP was prepared by centrifuging the blood twice for 10 minutes at 2630 g. The Coulter Counter analyzer (Beckman Coulter) was used for cell count in PRP.

Thrombin generation

TG was performed using the commercial CAT assay as previously published [7,24]. Briefly, 80 µl of plasma was added to 20 µl PRP reagent or 20 µl thrombin calibrator (Diagnostica Stago, France). After a 10 minute incubation in the device at 37°C, coagulation was activated by automatic dispensing 20 µl of FluCa reagent containing calcium and a fluorogenic substrate (Diagnostica Stago, France). Calibration was needed to correct for inner filter effect and plasma color, as well as to convert fluorescence data into a nM thrombin concentration. The conversion of the fluorescence data was done automatically by the CAT software and the obtained TG curves were used later for computational analysis for extraction of the prothrombin conversion curves using thrombin dynamics analysis. From the obtained TG curves the following parameters could be derived: lag time, time-to-peak, peak height, endogenous thrombin potential (ETP) and velocity index.

Plasma levels of antithrombin, fibrinogen and $\alpha_2$macroglobulin

Antithrombin, fibrinogen and α_2M levels were measured to perform thrombin dynamics analysis. Antithrombin was measured on the automated coagulation analyzer STA-R according to manufacturers specifications (Diagnostica Stago, France). Functional fibrinogen levels were measured using the Clauss method on the STart (Diagnostica Stago, France) [25]. Plasma α_2M levels were measured with an in-house chromogenic assay as previously described by Kremers et al [11].

Thrombin dynamics

The TG curve is the net result of prothrombin conversion and thrombin inactivation. Therefore, the course of prothrombin conversion can be calculated if TG and thrombin inactivation are known (Eq.1-3). Thrombin inactivation was determined by the previously described computational model which is based on the plasma antithrombin, α_2M and fibrinogen levels and the total inhibitory potential of each plasma was quantified by the thrombin decay constant (Eq.2; [26]). The values of K_{AT} and $K_{\alpha 2M}$ have been estimated previously [11].

Eq.1 $d(T)/dt = - d(P)/dt – d(T\text{-}inh)/dt$

Eq.2 $d(T\text{-}inh)/dt = K_{AT} \times [AT]_t \times [T]_t + K_{\alpha 2M} \times [\alpha_2M]_t \times [T]_t$

Eq.3 $- d(P)/dt = d(T)/dt + K_{AT} \times [AT]_t \times [T]_t + K_{\alpha 2M} \times [\alpha_2M]_t \times [T]_t$

Additionally, thrombin dynamics analysis was used to determine prothrombin conversion curves from TG. The following parameters could be quantified from the prothrombin conversion curves: PC_{tot} (total amount of prothrombin converted during the TG test), PC_{max} (maximum rate of prothrombin conversion), T-AT (amount of thrombin-antithrombin complexes formed) and T-α_2M (amount of thrombin-α_2M complexes formed) [26-28].

Statistics

Graphpad software was used to determine the statistical significance of the results. Outliers were checked using the ratio D/R. The results are presented as mean ± standard deviation (SD) and median ± inter-quartile range (IQR). Interindividual variability (%CV) was calculated for each variable as 100 × (standard deviation/mean). Reference ranges of PRP TG and thrombin dynamics parameters were calculated as 2.5^{th} to 97.5^{th} percentile according to the Clinical& Laboratory Standards Insitude (CLSI) guideline [29]. One-way ANOVA or Friedman analysis were used to detect differences depending on normality. Pearson or Spearman correlation test was used to determine correlations between parameters. P-values < 0.05 were considered statistically significant.

RESULTS

Thrombin generation in freshly isolated PRP relies on platelet count

To determine the role of the platelet count on TG, blood samples were collected from 117 healthy donors, of which 58 were men and 59 were women. The mean age of the subjects was 35 years (range, 18-65 years). The mean platelet count of whole blood and PRP were 241 x10^9/L (range 103-396 x 10^9/L)and 402 x 10^9/L (range 133-725*10^9/L), respectively. TG was measured in undiluted PRP, as well as in PRP diluted with autologous PPP to obtain a platelet concentration of 50 x 10^9/L and 150 x 10^9/L (Fig. 1). Mean ETP values (±SD) were 1412 (±252), 1502 (±271) and 1540 (±294) nM for PRP with a platelet count of 50 x 10^9/L, 150 x 10^9/L and original concentration (mean 402*10^9/L), respectively. Mean values (±SD) for peak height were 65 (±15), 95 (±21) and 135 (±28) nM thrombin and for velocity index 5 (±2), 9 (±3) and 20 (±7) nM/min in PRP with 50 x 10^9/L, 150 x 10^9/L and original platelet count, respectively. Table 1 shows mean, SD, CV, median, interquartile ranges (IQRs) and reference ranges of all TG parameters. The interindividual variability was 18.6% for lag time, 19.1% for ETP, 21.1% for peak height, 16.4% for time-to-peak and 33% for velocity index in undiluted PRP. The interindividual variation in PRP was not affected by the platelet count. In contrast to the thrombin peak and velocity index, that increased linearly with the platelet count, the ETP reached a maximum level already at a platelet count of 150*10^9/L. This indicates that a higher platelet count accelerates the rate of thrombin formation, but does not affect the total amount of thrombin formed.

Table 1: Reference ranges and interindividual variability of thrombin generation.

	Mean	SD	%CV	Median	IQR (25%-75%)	Reference ranges (2.5%-97.5%)
TG in PRP with original platelet count (mean 402*10^9/L)						
Lag time (min)	5.9	1.1	18.6	5.7	5.1-6.5	3.9-8.5
ETP (nm*min)	1540	294	19.1	1499	1344-1716	1061-2312
Peak height (nM)	135	28	21.1	132	115-151	89-202
Time-to-peak (min)	12.8	2.1	16.4	12.7	11.4-14.5	9.2-17.5
VelIndex (nM/min)	20.0	6.6	33	18.6	14.7-23.6	9.6-36.7
TG in PRP with 50*10^9 /L platelets						
Lag time (min)	8.1	1.4	17.3	8.0	6.9-8.9	5.4-11.4
ETP (nm*min)	1412	252	17.9	1368	1217-1550	1043-2114
Peak height (nM)	65	15	23.7	63	54-74	44-110
Time-to-peak (min)	23.1	4.5	19.6	22.9	19.8-26.3	16.0-34.1
VelIndex (nM/min)	4.7	1.8	39.0	4.1	3.4-5.7	2.0-9.6
TG in PRP with 150*10^9/L platelets						
Lag time (min)	7.6	1.4	18.4	7.4	6.7-8.6	5.1-10.9
ETP (nm*min)	1502	271	18.0	1460	1299-1655	1078-2178
Peak height (nM)	95	21	21.8	91	80-105	61-141
Time-to-peak (min)	18.8	3.1	16.7	18.4	16.6-20.8	13.1-26.4
VelIndex (nM/min)	8.9	3.1	34.6	8.3	6.9-10.7	4.0-16.9

Abbreviations: TG, thrombin generation; ETP, endogenous thrombin potential; Vellndex, velocity index.

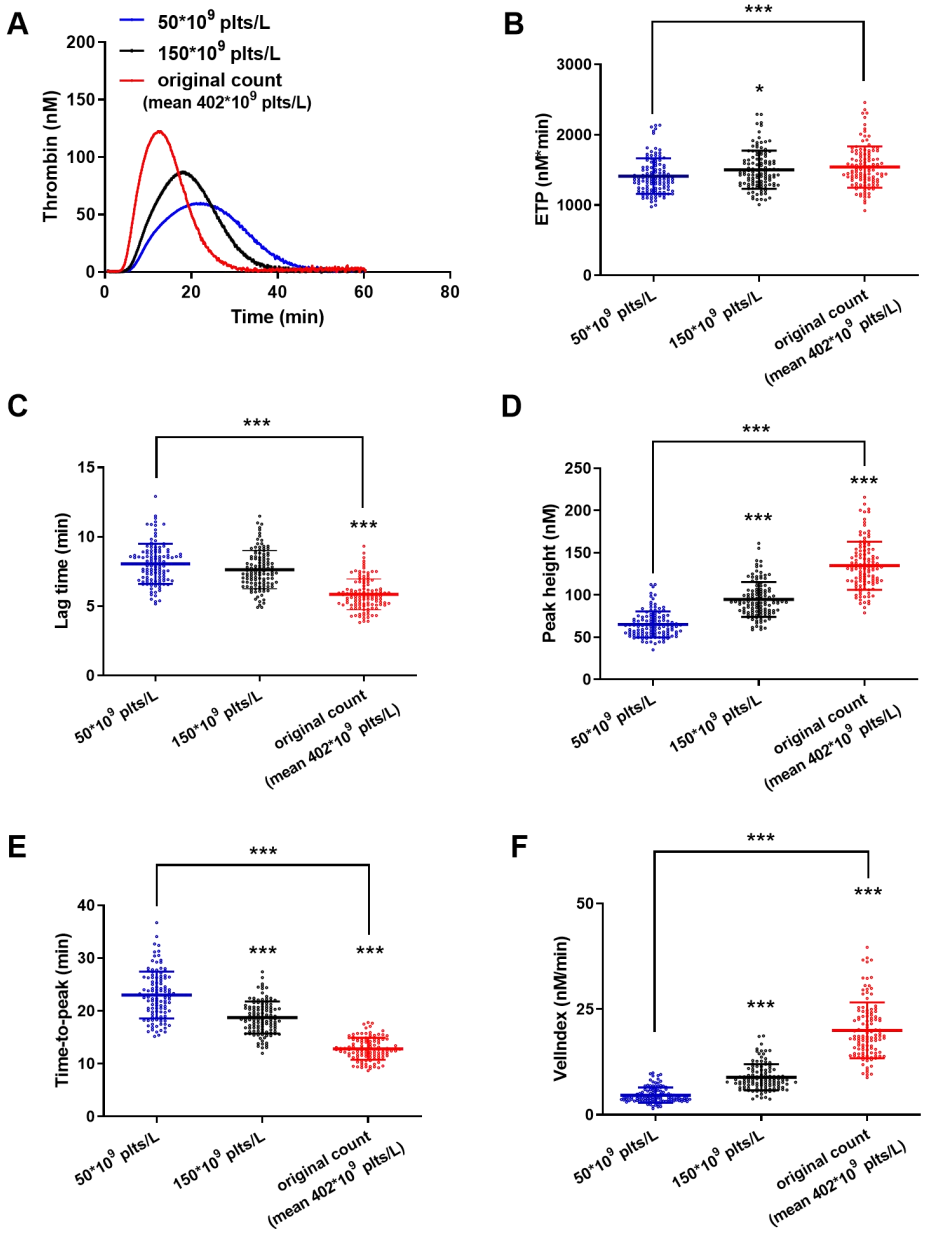

Figure 1: Effect of platelet count on tissue factor-induced thrombin generation in 117 healthy individuals. (A) Average thrombin generation curves at 1 pM TF were determined in PRP with a platelet count of 50*10^9/L (blue), 150*10^9/L (black) and original concentration (mean 402*10^9/L; red).

TG parameters ETP (B), lag time (C), peak height (D), time-to-peak (E), velocity index (VelIndex; F) were quantified. The data are shown as mean ± SD. Statistical significance was indicated as *p<0.05, **p<0.01, ***p<0.001 using ANOVA or Friedman analysis.

Influence of platelet count on thrombin dynamics

Plasma levels of fibrinogen, antithrombin and α_2M were measured to characterize coagulation factors levels in 117 healthy donors and to enable the calculation of thrombin dynamics (Table 2). The mean fibrinogen, antithrombin and α_2M plasma level were 2.88 g/L (±0.51 g/L), 2.72 µM (±0.19 µM) and 2.73 µM (±0.74 µM), respectively. To further investigate the effect of platelet count on thrombin formation, we determined the course of prothrombin conversion and thrombin inactivation during TG (Figures 2 & 3, Supplementary figure 2). When the plasma platelet count increased from 50 x 10^9/L to 150 x 10^9/L or to the original platelet count (mean 402 x 10^9/L), the total amount of prothrombin converted during TG also increased significantly (p<0.001). However, when the platelet counts reached 150 x 10^9/L, the total amount of converted prothrombin did not continue to increase significantly with increasing platelet count. Interestingly, the maximum rate of prothrombin conversion continued to increase with increasing platelet count, indicating that the rate of prothrombin conversion is highly dependent of the platelet count (p<0.001).

Table 2: Reference ranges and interindividual variability of coagulation factors and thrombin dynamics.

	Mean	SD	%CV	Median	IQR (25%-75%)	Reference ranges (2.5%-97.5%)
PRP original count	402	101	25.1	394	339-465	201-673
Fibrinogen (g/L)	2.88	0.51	17.7	2.85	2.58-3.17	1.93-4.14
Antithrombin (µM)	2.72	0.19	6.9	2.71	2.59-2.83	2.37-3.14
α_2M (µM)	2.73	0.74	27.6	2.55	2.16-3.14	1.42-4.42
Thrombin dynamics in PRP with original platelet count (mean 402*10^9/L)						
PC_{tot} (nM)	1159	152	13.1	1148	1052-1262	822-1504
PC_{max} (nM/min)	153	39	25.5	146	129-171	99-302
T-AT (nM)	1106	141	12.7	1091	1009-1210	775-1382
T-α_2M (nM)	41	13	31.8	39	31-48	21-74
TDC (min^{-1})	0.82	0.08	9.7	0.82	0.77-0.87	0.66-0.99
Thrombin dynamics in PRP with 50*10^9/L platelets						
PC_{tot} (nM)	985	157	15.9	1001	887-1079	645-1278
PC_{max} (nM/min)	102	23	22.7	99	85-114	67-158
T-AT (nM)	801	163	20.4	808	699-905	424-1142
T-α_2M (nM)	27	10	37.1	25	20-33	13-50
Thrombin dynamics in PRP with 150*10^9/L platelets						
PC_{tot} (nM)	1124	146	13.0	1105	1033-1237	885-1442
PC_{max} (nM/min)	124	27	21.4	121	105-133	88-201
T-AT (nM)	1009	139	13.8	1007	922-1110	698-1279
T-α2M (nM)	36	12	32.7	34	28-43	19-69

Abbreviations: PC_{tot}, total amount of prothrombin converted; PC_{max}, maximum rate of prothrombin conversion; T-AT, thrombin and antithrombin complex; T-α_2M, thrombin and α_2M complex.

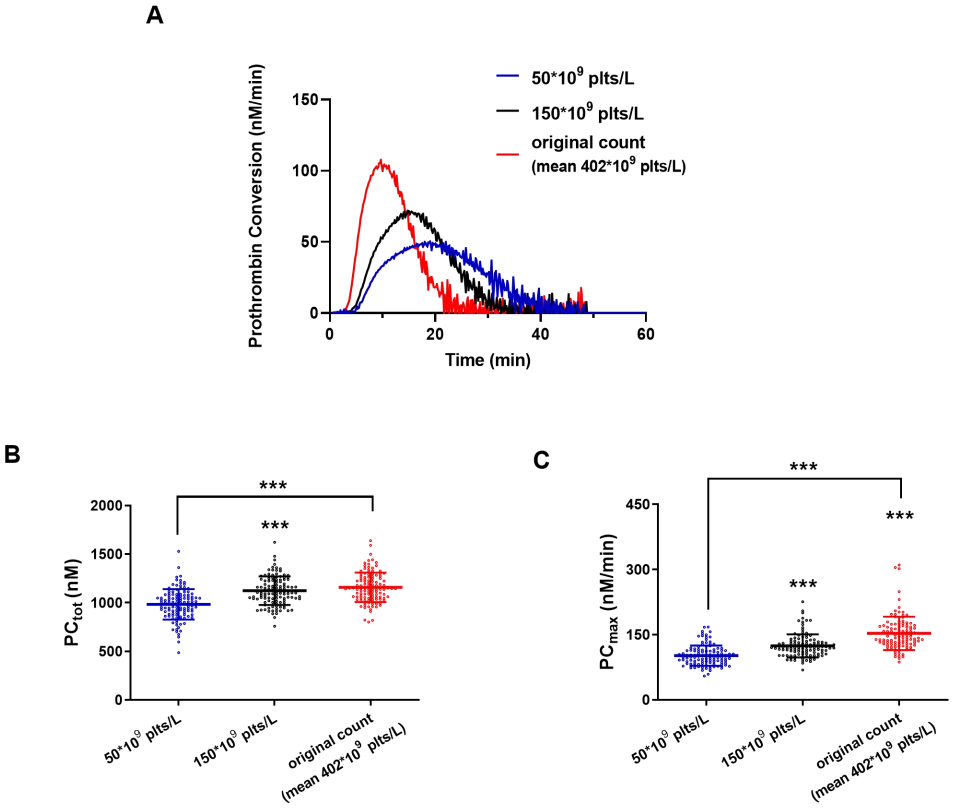

Figure 2: Effect of platelet count on prothrombin conversion in 117 healthy individuals. (A) Average prothrombin conversion curves in PRP with a platelet count of 50 x 10^9/L (blue), 150 x 10^9/L (black) and original concentration (mean 402 x 10^9/L; red). The prothrombin conversion parameters total amount of prothrombin converted (PC_{tot}; B) and maximum rate of prothrombin conversion (PC_{max}; C) were quantified from the prothrombin conversion data. The data are shown as mean ± SD. Statistical significance was indicated as ***p<0.001 using ANOVA or Friedman analysis.

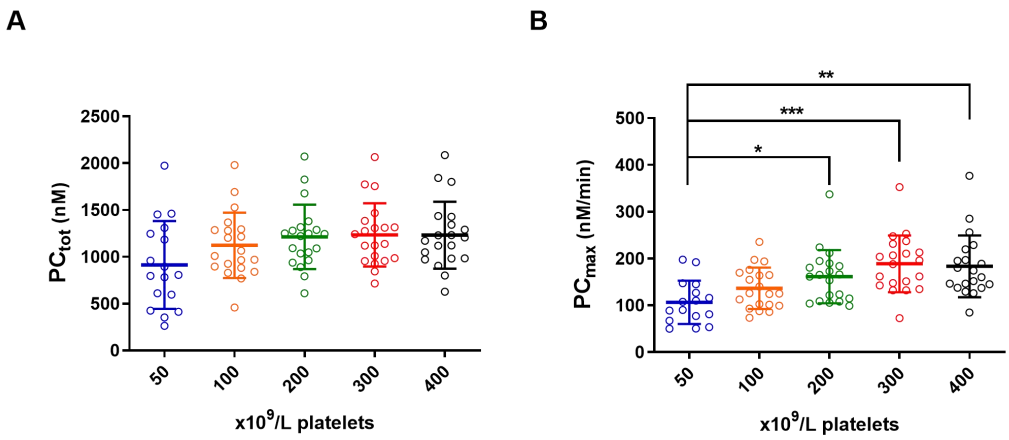

Figure 3: Effect of dose-response platelet count (range, 50-400*10^9/L) on thrombin dynamics in 20 healthy individuals. Total amount of prothrombin converted (PC_{tot}; A) and maximum rate of prothrombin conversion (PC_{max}; B) were quantified from the prothrombin conversion data. The data are shown as mean ± SD. Statistical significance was indicated as *p<0.05, **p<0.01, ***p<0.001 using Friedman analysis.

This finding was further investigated by obtaining a broader platelet dose-response of 50, 100, 200, 300 and 400 x 10^9/L in a small subgroup of the population (n=20, Figure 3). This subgroup consisted of 20 healthy donors, of which 9 were men and 11 were women. The mean age of the subjects was 40 years (range 19-65 years). The mean platelet count of whole blood and platelet rich plasma were 323 x 10^9/L (range 268-379) and 503 x 10^9/L (range 403-702), respectively. Prothrombin conversion (Figure 3A) increased up to 200 x 10^9/L and stabilized hereafter, while the velocity of prothrombin conversion (Figure3B) continued to increase significantly up to 300*10^9/L. Supplemental figure 1 and 2 show the effect of a broader platelet dose-response (10, 20, 25, 30, 40, 50, 100, 150, 200, 300 x 10^9/L and undiluted) on TG and thrombin dynamics, respectively. When the platelet count is between 10 x 10^9/L to 40 x 10^9/L, the ETP, peak height, PC_{tot} and PC_{max} showed an increasing trend. More interestingly, peak height and velocity index started to increase sharply in PRP from a platelet count of 50 x 10^9/L.

The rate of thrombin inactivation can be quantified by the thrombin decay capacity (TDC), which is the pseudo-first order decay content for thrombin inhibition by plasmatic thrombin inhibitors, including antithrombin and α_2M and plasma fibrinogen level. As shown in figure 4, the mean thrombin decay capacity constant is 0.82. The inhibition of thrombin by antithrombin and α_2M, leads to the formation of the complexes T-AT and T-α_2M. Both complexes increased significantly with increasing platelet count. However, the ratio of the contribution of T-AT and T-α_2M complexes to the total amount of inhibited thrombin did not alter with increasing platelet count. Table 2 shows mean, SD, CV, median, interquartile ranges (IQRs) and the reference ranges of all thrombin dynamics parameters. The interindividual variation of PC_{tot}, PC_{max}, T-AT and T-α_2M were 13.1%, 25.5%, 12.7% and 31.8%, respectively.

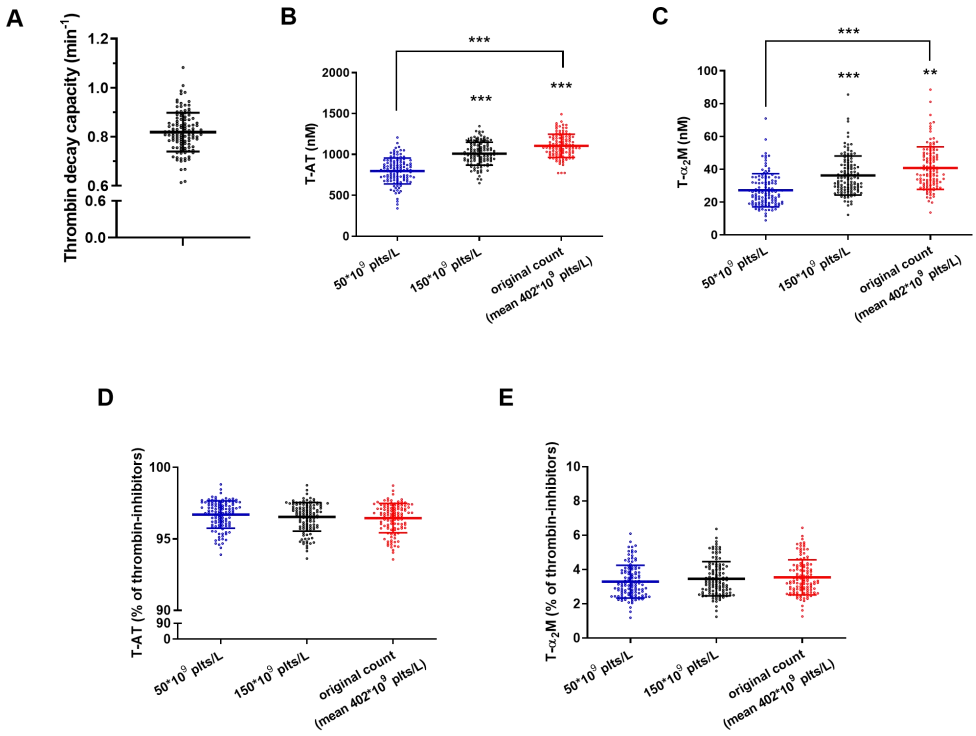

Figure 4: Effect of platelet count on thrombin inactivation in 117 healthy individuals. (A) The thrombin decay capacity assessed in 117 healthy individuals was based on plasma AT, α_2M and fibrinogen levels. (B) T-AT complexes formed during thrombin generation. (C) T-α_2M complexes

formed during TG. (D, E) Contribution of T-AT and T-α_2M to total thrombin inhibition. The data are shown as mean ± SD. Statistical significance was indicated as ** p<0.01, ***p<0.001 using ANOVA or Friedman analysis.

Correlation of thrombin generation, prothrombin conversion and thrombin inactivation

Correlation analysis of TG, prothrombin conversion and thrombin inactivation revealed that platelet count was associated with lag time (r=0.213), peak height (r=0.329) and velocity index (r=0.229) in TG (Table 3). No significant correlation could be found between platelet count and ETP or time-to-peak. However, platelet count did correlate significantly with the total amount of prothrombin converted (r=0.184), the maximum rate of prothrombin conversion (r=0.300) and T-AT complex (r=0.224) during thrombin formation, but not with T-α_2M. In this study, platelet count mainly contributed to the rate of prothrombin conversion during thrombin formation.

Table 3: Correlation coefficients between platelet count and parameters.

TG			PC			TDC		
	r	**p value**		**r**	**p value**		**r**	**p value**
Lag time	0.213	0.021	**PC_{tot}**	0.184	0.047	**T-AT**	0.224	0.016
ETP	ns	0.159	**PC_{max}**	0.300	0.001	**T-α_2M**	ns	0.325
Peak	0.329	<0.001				**TDC**	ns	0.461
TTP	ns	0.794						
VelIndex	0.229	0.014						

Abbreviations: TG, thrombin generation; PC, prothrombin conversion; TDC, thrombin decay capacity; ETP, endogenous thrombin potential; TTP, time-to-peak.

DISCUSSION

The goal of this study was to establish the reference ranges of TG and thrombin dynamics in PRP of 117 healthy donors, as well as to determine the effect of platelet count on the thrombin dynamics in these healthy donors. Previously, Bloemen et al. described in 2017 the interindividual variation and normal ranges of TG in PPP, PRP and whole blood in a large population of healthy donors [30]. However, the use of in-house reagents and presenting the data as normalized values makes it difficult for other laboratories to compare their data. Therefore, for this study, it was decided to use the CAT assay of Diagnostica Stago with the corresponding commercially available reagents in order to obtain reference values that all laboratories can use to compare their data when using the same assay. Published studies have shown acceptable reproducibility with an interindividual variability of 16-35% in PRP TG [18,30]. In our study, the value of the parameters was influenced by platelet count, but the interindividual variability of TG and thrombin dynamics was consistent between 13-39% (Table 1 and 2).

In the past, the important contribution of platelets to the clotting systems could be demonstrated by performing TG in PRP [31-33]. Schols et al. showed that platelets can partly compensate for the dilution effect [34]. Additionally, as activated platelets expose phosphatidylserine (PS) at their surface to sustain prothrombinase activity, it was shown that blocking the PS led to an inhibition of TG [35]. In this study, the contribution of platelets to TG was investigated more in dept by performing thrombin dynamics analysis in PRP with varying platelet concentrations by diluting PRP with autologous PPP. From this analysis it could be concluded that increasing the platelet concentration predominantly affected the rate of prothrombin conversion and TG, rather than the total amount of TG. This was shown by the thrombin peak height and velocity index, that increased significantly with increasing platelets. This finding was also confirmed by correlation analysis, where the platelet count correlated significantly with peak height and velocity index in TG (Table 3). However, the ETP, which is a measure for the total amount of active thrombin formed, increased and reached a plateau value already at a platelet count of 150 x 10^9/L (Fig. 1). Similarly, in the prothrombin conversion process, the total amount of prothrombin converted increased with the platelet count, but also reached a maximum rate

at 150 x 10^9/L (Fig. 2). A possible explanation for this could be that when the platelet count is low, the exposed PS on the platelet surface is not sufficient to convert all prothrombin. When the platelet count increases this results in a higher conversion of the available prothrombin. Earlier, it was also shown that the more procoagulant surface is available, the faster prothrombin can be converted, resulting in an elevated peak height and velocity index [11]. The ETP, in contrast, is affected considerably less by the rate of thrombin production, and mainly depends on the amount of thrombin formed and the capacity of thrombin inhibitors to clear the plasma from active thrombin. Therefore, increasing the platelet count above 150*10^9/L will not affect the ETP any more, although it will affect the velocity of TG and therefor the peak height and velocity index will still increase.

Additionally, another possible explanation for this phenomenon is that activated platelets not only provide the phospholipid membrane on which TG can take place, but platelets are also involved in the production of certain coagulation factors (e.g. fibrinogen, FV, VWF and FVIII) [33,34,36]. The release of platelet content upon platelet activation can also affect TG. For instance, 20% of the circulating factor V (FV) is located in α-granules and FV contributes to an increase in the prothrombin conversion velocity [37]. Moreover, VWF is also stored in platelet α-granules (20%) and it is known that VWF can not only bind and transport factor VIII (FVIII), but it can also activate platelets [38]. Therefore, the more platelets, the more FV and VWF will be released, leading to an increase in TG.

The supplemental figures show the effect of a broader platelet dose-response on TG and thrombin dynamics. In these dose-response curves, we also included samples with a very low platelet concentration, in the range of 10 x 10^9/L to 50 x 10^9/L. Such low platelet concentrations are also seen in patients with thrombocytopenia, which is a common, but serious condition [39-41]. Mucocutaneous bleeding usually occurs when the platelet count decreases to a very low range of 20 x 10^9/L to 30 x 10^9/L. Furthermore, severe bleeding, as e.g. intracranial hemorrhage, occurs when the platelet count is in a range of 10 x 10^9/L to 20 x 10^9/L [42,43]. We could conclude from our experiments that when the platelet count was below 50 x 10^9/L, the slopes of the dose-response curves of the TG parameters were not steep, however, they became steeper at platelet count of 40 x 10^9/L to 50 x 10^9/L. Suggesting that this platelet count may be a turning point for TG and that when the platelet count is below this level, the platelets fail to adequately support thrombin generation. Moreover, even if the prothrombin concentrations of our plasma samples were unchanged, we did observe that when the platelet count decreased, the total amount and the rate of

prothrombin conversion decreased as well.

Thrombin inactivation was quantified in two ways: (i) The total decay capacity describes the rate at which a fixed amount of thrombin is neutralized in plasma and depends on the plasma fibrinogen, antithrombin and α_2M level. The decay capacity of antithrombin and α_2M are affected by fibrinogen, mainly due to the binding of thrombin to fibrinogen, which interferes with the binding of its inhibitors [16]. (ii) The T-AT and T-α_2M complexes are formed during TG, the amount of which depends on the amount of prothrombin converted (i.e. thrombin produced) (Figure 4) [11]. The ratio of thrombin inhibition by antithrombin or α_2M remains the same, independently of the total amount of thrombin formed. This was also shown previously in PPP when the α_2M and AT levels were normal [15].Our findings show that the presence of platelets does not change the kinetics of thrombin inhibition by either AT or α_2M, as the thrombin decay capacity is unaltered and the balance between T-AT and T-α_2M is unchanged.

In conclusion, we established the reference ranges of TG and thrombin dynamics of PRP. In addition, we investigated the influence of a varying platelet count on thrombin kinetics. Our results indicated that higher platelet counts mostly affected the rate of prothrombin conversion and TG rather than the total amount of thrombin formed.

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DECLARATION OF INTEREST STATEMENT

All authors report no conflicts of interest.

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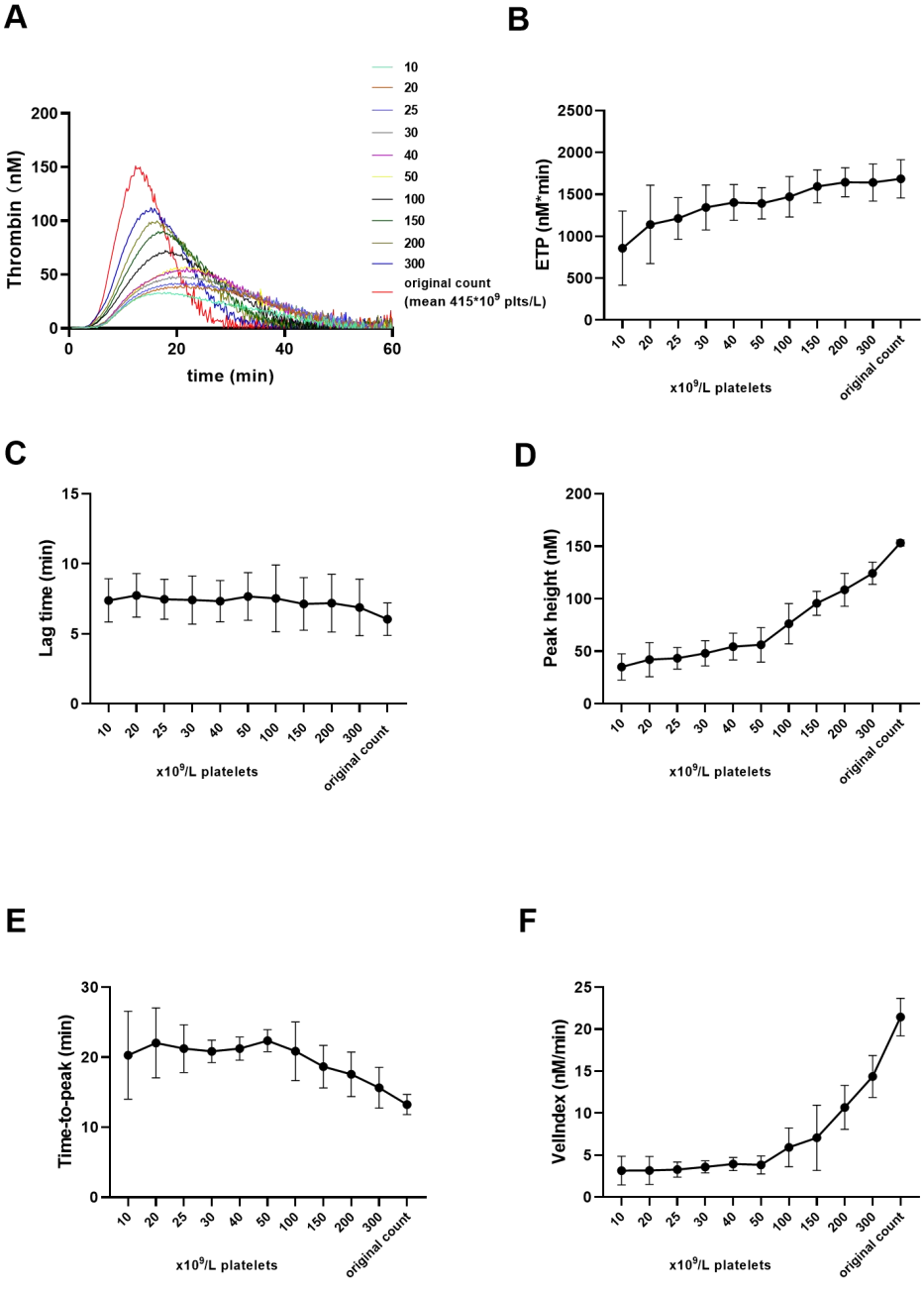
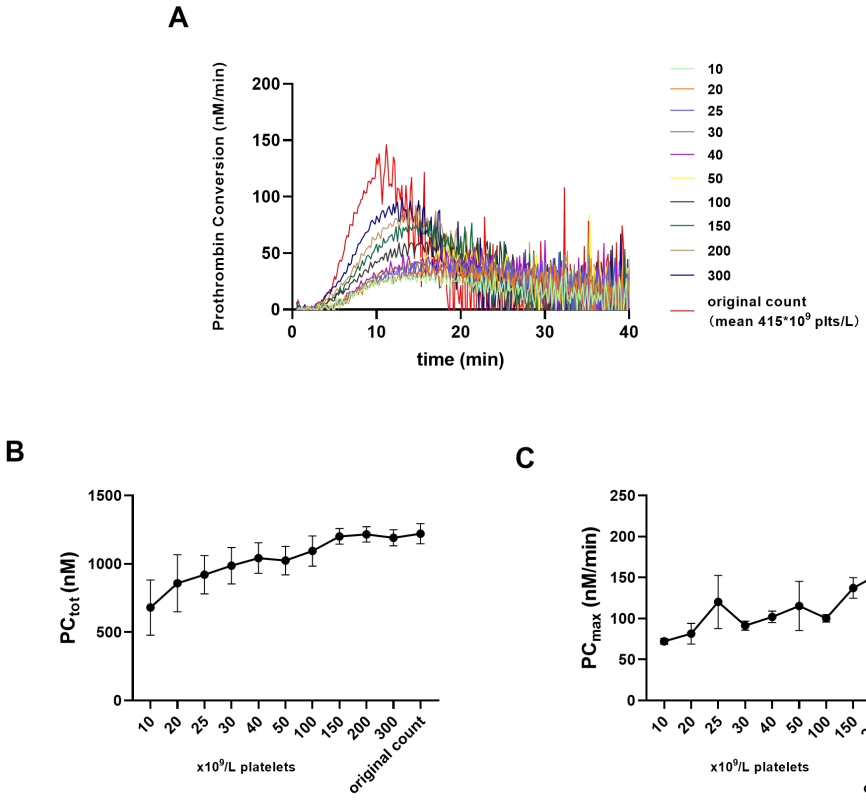
SUPPLEMENTARY MATERIAL



Supplementary figure 1: Effect of a platelet dose-response (range, 10-415*10^9/L) on thrombin generation in 3 healthy individuals. Average TG curves measured with 1 pM TF were determined in PRP with a varying platelet count (A). ETP (B), lag time (C), peak height (D), time-to-peak (E) and velocity index (VelIndex; F) were quantified. The data are shown as mean ± SD.



Supplementary figure 2: Effect of a platelet dose-response (range, 10-415*10^9/L) on thrombin dynamics in 3 healthy individuals. Averaged prothrombin conversion curves in PRP with a varying platelet count (A). Total amount of prothrombin converted (PC_{tot}; B), maximum rate of prothrombin conversion (PC_{max}; C) during TG were quantified from the prothrombin conversion data. The data are shown as mean ± SD.

Chapter 6

General discussion & Summary

GENERAL DISCUSSION

The thrombin generation (TG) assay is a global coagulation assay that depends on the pro- and anticoagulant processes of prothrombin conversion and thrombin inactivation [1-6]. Many studies have shown an association of TG parameters with bleeding [7-10] or thrombosis [11-14]. TG can has been used to study the severity of hemophilia A patients and monitor their therapy [15,16], or to determine the optimal dose of anticoagulants for patients individually [17,18]. Although TG is a very useful test to assess overall haemostasis, the global nature of this test makes it difficult to pinpoint recorded changes in coagulation to a single coagulation factor or process. Thus, an increase in TG can be caused by an elevated thrombin production (increased prothrombin conversion) or a reduced thrombin inactivation, causing thrombin to accumulate (Figure 1) [19]. Vice versa, a low TG can be attributable to a reduction of prothrombin conversion or an increased thrombin inactivation [20,21]. Antithrombin (AT) and α_2-macroglobulin (α_2M) are the important natural thrombin inhibitors. In complex coagulopathies, both the pro- and anticoagulant pathways can be affected simultaneously, which can either lead to a complex coagulopathy or to a rebalanced TG profile [22].

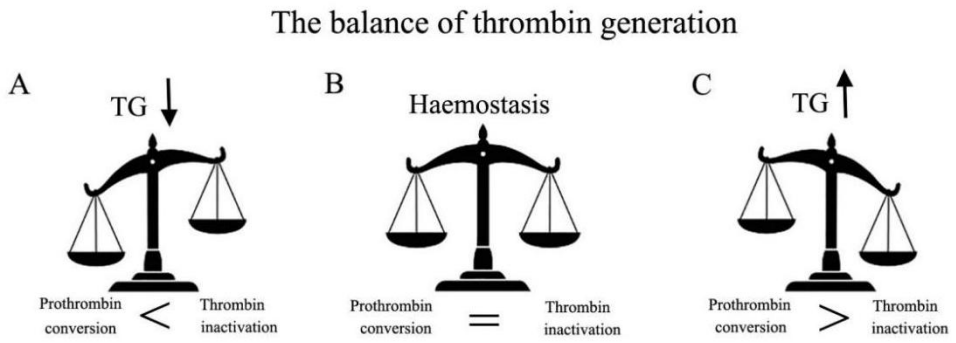


Figure 1: The balance of prothrombin conversion and thrombin inactivation during thrombin generation. (A) A lower TG can be caused by a reduction of prothrombin conversion or an acceleration of thrombin inactivation or both. (B) Hemostasis is the balance between prothrombin conversion and thrombin inactivation. (C) An elevation in TG can be caused by a reduction of

thrombin inactivation or an increase of prothrombin conversion or both (figure adapted from ref [23]).

The thrombin dynamics analysis method was developed to investigate the main pro- and anticoagulant processes that underly TG in more detail. Kremers et al. developed the computational thrombin dynamics approach to study prothrombin conversion and thrombin inactivation during the measurement of TG [20,23,24]. Thrombin dynamics analysis was previously used for the analysis of TG curves measured in platelet-poor plasma (PPP). Several clinical studies have shown its suitability for finding explanations for altered TG profiles in multiple pathologies [20,25]. Figure 2 summarizes the clinical and basic studies using thrombin dynamic analysis over the last decade. Previously published data are depicted in blue and the studies presented in this thesis are shown in orange.

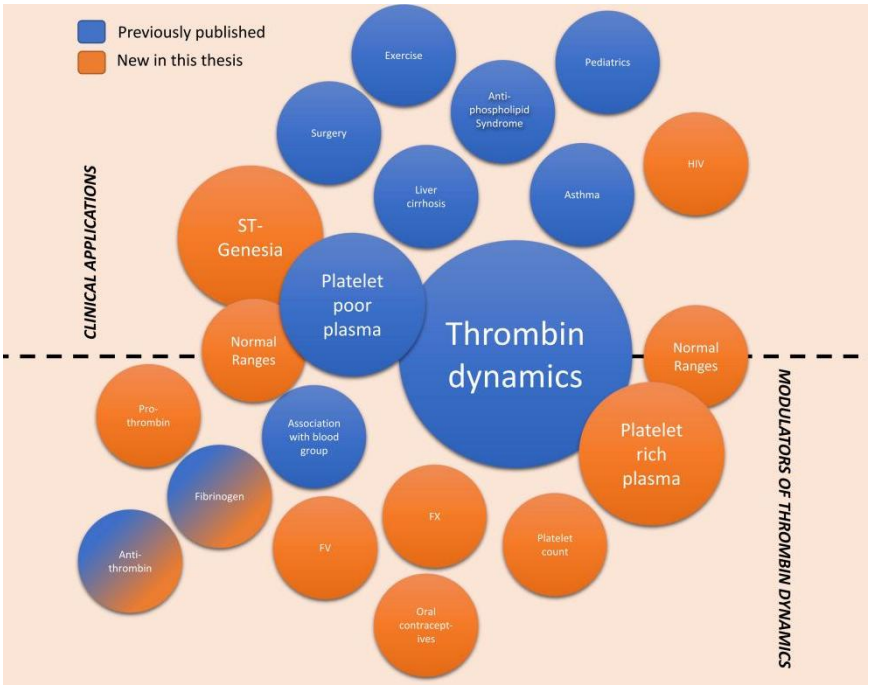


Figure 2: Overview of studies using thrombin dynamics analysis in the past (blue) and new studies discussed in this thesis (orange).

Several aspects of the further development of the thrombin dynamics method are described in this thesis, including (1) the determination of reference values to provide guidance on how to interpret 'normal' vs. 'abnormal' test results, (2) the use of the method in platelet-rich plasma, (3) the further assessment of the clinical usefulness of the method in a group of HIV positive individuals, and (4) the preparation of the method for its use in a clinical and diagnostic laboratory setting. We discuss these aspects in further detail below.

'NORMAL' PROTHROMBIN CONVERSION AND ITS CLINICAL MEANING

Now that the thrombin dynamics method has been clinically validated in several patient populations [20,23-25], it is important to determine which are the normal and abnormal thrombin dynamics test results. The reference values reported in Chapter 4 can be used as guidance for clinicians to interpret the relevance of the changes in prothrombin conversion, and to bring the measurement of prothrombin conversion a step closer to its use in the clinical laboratory.

In the past, the detection of residual prothrombin levels in serum, which was called prothrombin consumption test, was used to diagnose several clotting and platelet disorders. Use of this early test indicated that the assessment of prothrombin conversion is beneficial for the diagnosis of hemostatic disorders [26,27]. First studies using thrombin dynamics analysis have shown that indeed patients with haemostatic defects in the generation of thrombin, such as hemophilia A patients (Chapter 4), have a reduced rate of prothrombin conversion (PC_{max}) and a reduced amount of prothrombin converted throughout TG (PC_{tot}) compared to healthy individuals. Thus, in healthy individuals, PC_{max} ranges from 109 nM/min to 415 nM/min, whereas the average PC_{max} in hemophilia A patients is 27 nM/min. Similarly, PC_{tot} ranges from 693 nM to 1344 nM in healthy individuals, whereas the average PC_{tot} in hemophilia A patients is 330 nM. Although similar conclusions were drawn in the initial hemophilia A study concerning a marked reduction of prothrombin conversion in plasma from patients compared to controls, the new reference values simplifies the detection of sub- or super-normal prothrombin conversion in patients with presumed

haemostatic defects.

MODULATORS OF PROTHROMBIN CONVERSION

Prothrombin conversion is known to be affected by many factors, including the plasma prothrombin level, the amount and kinetics of the prothrombinase complex present in the clotting plasma and the number of platelets [28]. In thrombin dynamics analysis, two parameters are used to quantify prothrombin conversion: PC_{tot} and PC_{max}. From our studies we can conclude that PC_{tot} is mainly dependent on prothrombin and FX levels in plasma (Chapter 2). In contrast to PC_{tot}, PC_{max} is not only dependent on the plasma prothrombin and FX levels, but also on the antithrombin (AT) level [10].

Prothrombin

In-vitro dose-response experiments demonstrated that the prothrombin level determines the PC_{tot} and PC_{max}. This is also expected based on the laws of enzyme kinetics, as the velocity of an enzymatic reaction increases when the amount of substrate of the enzyme, in this case prothrombin, increases [29]. A clinical example of the impact of prothrombin concentration in the process of prothrombin conversion is the low prothrombin conversion in children that is caused by lower plasma prothrombin levels compared to adults [30]. On the other hand, elevated prothrombin levels and consequently a higher prothrombin conversion, are associated with thrombotic disorders, particularly venous thrombosis. For example, the prothrombin gene mutation *G20210A* is associated with a higher plasma prothrombin level, and also with an increased risk of developing deep vein thrombosis and pulmonary embolism [31].

Upstream coagulation factors

Nevertheless, changes in prothrombin conversion are not always caused by changes in plasma prothrombin levels, but also by changes in the levels of the coagulation factors

more upstream in the coagulation pathway. For example, in Chapter 3 of this thesis we show that the prothrombin conversion is increased in HIV patients using abacavir, independently of the plasma prothrombin level. The changes can, for example, alter the efficiency of the prothrombinase complex (e.g. by altered FV or FX levels) or the tenase complex (e.g. by altered FVIII or FIX levels). The latter occurs in haemophilia A and B, in which cases a reduced prothrombin conversion is attributed to lower levels of FVIII or FIX and is associated with a bleeding phenotype [29,32]. Patients suffering from FV deficiency usually present with a bleeding tendency of variable severity, which is reflected in a prolonged PT and APTT, as well as in a prolonged TG lag time [33-35]. Using thrombin dynamics analysis, full FV deficiency results in a severe reduction of PC_{tot} and $PC_{max.}$ However, above a FV level of 10%, PC_{tot} and PC_{max} reach a plateau level with a similar value as obtained in plasma containing 100% FV. This finding is in line with reports that very low levels of FV are sufficient to support hemostasis.Additionally,FX deficient patients tend to have severe symptoms, similar to those of a FVIII and FIX deficiency [34,36-38]. In this thesis we show that the plasma level of FX influences the prothrombin conversion by affecting the rate of this conversion [29]. Specifically, PC_{max} increases dose-dependently with the FX level[29]. Additionally, higher FX increases PC_{tot}, T-AT and T-α_2M, until a plateau is reached when FX levels are above 40%. This indicates that, in physiological circumstances, sufficient FX is available to sustain a normal haemostasis. Together with prothrombin and FV, FX is a main rate-limiting factor for the conversion of prothrombin. A lower FX, especially in combination with lower prothrombin and/or FV levels, then results in a significant reduction of PC_{max}. A clinical example of this situation can be found in patients using vitamin K antagonists (VKA). VKA therapy is for example used to reduce the risk of thrombosis recurrence in the antiphospholipid syndrome (APS), because intake of VKA reduces the plasma levels of prothrombin and FX, thereby reducing the conversion of prothrombin to thrombin, which may be critical for preventing thrombosis in APS. The results of my thrombin dynamics analysis also showed that VKA therapy reduced the PC_{max} in APS patients[25].

Platelets

The activation of platelets and subsequent phosphatidylserine expression is an important step in blood-based coagulation. Phosphatidylserine-exposing platelets provide a procoagulant surface that is necessary for the assembly and function of the prothrombinase and tenase complexes [39-41]. Additionally, platelets secrete certain coagulation factors, such as fibrinogen and FV [42]. The release of granular content upon platelet stimulation appears to influence TG, as, for example, 20% of the circulating FV is located in the α-granules and FV exhibits substantial cofactor activity to increase prothrombin conversion [42]. Additionally, VWF is stored in the α-granules. It is known that VWF does not only stick platelets, but also binds and transports the FVIII in the blood [41,43].

In this thesis I describe how thrombin dynamics analysis can be performed in platelet-rich plasma (Chapter 5). Accordingly, this analysis can now also be used to investigate the involvement of platelets in thrombotic and bleeding disorders [44]. We studied the influence of platelet count on TG, prothrombin conversion and thrombin inactivation. Platelet count dose-dependently increased the TG peak and velocity by enhancing the rate of prothrombin conversion (PC_{max}) in a dose-dependent manner. This is in line with a report of Quick et al., showing that the rate of prothrombin consumption increases proportionately with the platelet count until a plateau of prothrombin consumption is reached [45]. Additionally, the total amount of thrombin that was converted (PC_{tot}) increased dose-dependently with the platelet count, until a plateau was reached at approximately 150 x 10^9 platelets/L (Chapter 5).

Literature reports show that thrombocytopenia is associated with a lower TG and with mild to severe bleeding events[46]. The validation of the thrombin dynamics method for PRP (Chapter 5) provides a new opportunity to study the effect of low platelet count on TG in more detail. A future opportunity would be to investigate how TG and thrombin dynamics parameters change in response to the various platelet agonists.

'NORMAL' THROMBIN INACTIVATION, ITS MODULATORS AND ITS CLINICAL MEANING

Once thrombin is formed *in vivo*, it is rapidly neutralized by thrombin inhibitors to prevent continuous clotting. Insufficient natural anticoagulant action, for example caused by antithrombin (AT) deficiency, can result in thrombosis [47-49]. Vice versa, if thrombin inactivation occurs too fast, for example in patients treated with heparins, which accelerate the binding of thrombin and AT, bleeding can occur[50,51]. This inactivation of thrombin is another important and tightly regulated process in haemostasis.

AT is the main natural inhibitor of thrombin in plasma, and plasma AT levels strongly influence the thrombin decay capacity [47-49]. Decreasing the AT level causes a dramatic increase in TG by reducing the thrombin decay capacity (TDC), which is in line with the *in vivo* consequences of AT deficiency. Interestingly, we find that the effect of AT levels on T-AT are different in physiological and pathological states. In healthy individuals, AT levels did not alter the formation of T-AT complexes, which may be due to the excess amount of AT relative to the amount of thrombin present. However, under pathological conditions, such as liver disease, insufficient synthesis of AT leads to a decrease in T-AT complexes. In chronic liver disease and young children, functional AT levels are lower compared to healthy adults and α_2M levels are increased [30,54]. Therefore, the relative contribution of AT to the inhibition of TG is lower in children and, in combination with increased α_2M levels, this causes the balance between T-AT and T-α_2M to shift towards more T-α_2M [55,56].

Another important modulator of thrombin inactivation is fibrinogen (and fibrin fibers during clot formation), which binds thrombin and hampers its inactivation by AT and α_2M [20,24]. In contrast to the profound effect of platelets on prothrombin conversion, an altered platelet count does not change the kinetics of thrombin inhibition by either AT or α_2M, as the thrombin decay capacity is unaltered and the balance between T-AT and T-α_2M is unchanged. Our results suggest that platelets mainly influence the procoagulant processes rather than the inactivation of thrombin.

In the thrombin dynamics analysis, thrombin inactivation is quantified in two ways.

The formation of T-AT and T-α_2M complexes during TG is quantified and appears to be dependent on the amount of prothrombin converted. Additionally, the thrombin decay capacity (TDC) quantifies the total capacity of an individual plasma to inhibit thrombin, independent of the rate and amount of prothrombin converted [29]. This makes the decay capacity independent of prothrombin conversion and allows comparison between subjects without the interference of their TG potential. For example, in Chapter 3 we show that that tenofovir disoproxil fumarate (TDF) in HIV positive individuals resulted in a higher thrombin inactivation due to higher AT levels independently from the prothrombin conversion, which led to a lower TG. This is in line with other reports that HIV patients treated with TDF have a lower or similar risk of developing thrombotic events [57-59].

BRINGING THROMBIN DYNAMICS TO THE CLINIC

As mentioned above, the establishment of reference values for thrombin dynamics parameters is a step forward in the use of the prothrombin conversion test in clinical and diagnostic laboratories. However, a hurdle to overcome is the fact that the semi-automated TG test as measured on the CAT device is not suitable for use in a routine clinical laboratory. The newly developed fully-automated ST-Genesia is now available to clinical labs for the measurement of TG. Therefore, we adapted the thrombin dynamics analysis method for use in combination with the ST-Genesia (Chapter 4).In my thesis, we present the reference ranges in 112 healthy controls for thrombin dynamics parameters using the ST Genesia, which now can be used to detect discrepancies between healthy subjects and patient populations in clinical and research studies. We demonstrated that thrombin dynamics parameters did not differ between men and women without oral contraceptive use and the use of oral contraceptive causes an increase in thrombin dynamics parameters, regardless of the presence of thrombomodulin. These findings are in line with the pro-coagulant effect of oral contraceptive use described in literature and previous studies performed using the CAT device [60,61]. Therefore, the introduction of the ST Genesia into the clinic is an opportunity to use the thrombin dynamics analysis in specified clinical settings

in the future.

A specific clinical field in which the automated measurement of TG and thrombin dynamics would be useful is the haemophilia field. Recent advances in technology have resulted in a novel therapeutic approach for the prophylactic treatment of haemophilia by rebalancing the coagulation system through antithrombin targeting [62]. For example, Fitusiran is an RNAi therapeutic targeting AT, which restores TG and rebalances hemostasis in haemophilia A or B patients [63]. In Chapter 2 and in later studies of our group we showed that thrombin dynamics analysis could be used to mimic the effect of AT lowering in silico on TG and thrombin dynamics in hemophilia patients [64]. By estimating the effect on the thrombin dynamics parameters of prothrombin conversion and thrombin inactivation, the pre-therapeutic dose of Fitusiran can be adjusted in the future for each individual patient.

FUTURE PERSPECTIVES

Thrombin dynamics analysis is currently only available for plasma-based TG assays. Therefore, the effect of erythrocytes, leukocytes, and other components that are filtered from whole blood when preparing (platelet-rich) plasma, are currently not taken into account. The measurement of TG in whole blood was more complicated than TG in plasma, because erythrocytes distort the fluorescence signal transmission during the TG measurement [65]. Recently efforts have been made to simplify the protocols for TG in whole blood, which is expected to make the method of interest for a broader range of researchers [65,66]. A logical next step would be to re-design thrombin dynamics analysis for TG measured in whole blood.

CONCLUSION

In this thesis, I have re-investigated the thrombin dynamics method in substantial depth. Thrombin dynamics analysis now appears to extract additional information from the underlying TG by analyzing the pro- and anticoagulation processes in a wide variety of plasma samples. I established reference values of thrombin dynamics parameters in

platelet-poor and platelet-rich plasma.Furthermore, I used thrombin dynamics to better understand the changes in coagulation in HIV and haemophilia A patients. Although more studies are needed, the present results indicate that the test of plasma-thrombin dynamics has promising applications in the laboratory and in clinic. Additionally, in this thesis, I made a first step towards automated clinical assessment of thrombin dynamics for its implementation in patient care.

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SUMMARY

The thrombin generation (TG) assay is a global coagulation assay used in research and in clinical settings. The advantage of measuring TG is that various coagulation abnormalities can be detected with this assay, that can result in an increased risk for developing a bleeding or thrombosis. This is in contrast to the conventional tests, such as the clotting times routinely used in the clinic, which, for example, cannot shorten and therefore cannot detect an increased risk of thrombosis. However, some specific coagulation abnormalities cannot be identified by TG alone. Kremers et al. developed a new method to investigate the main pro- and anticoagulant processes underlying TG through computational analysis of the dynamics of thrombin formation. By applying the thrombin dynamics method, one can obtain the course of prothrombin conversion into thrombin, and of the thrombin inactivation, which occur both during TG. The thrombin dynamics analysis has been described previously for analyzing TG measured in platelet-poor plasma, and several clinical studies have demonstrated its utility for explaining altered TG profiles in various pathologies. In this thesis, we further investigated the clinical relevance of thrombin dynamics in platelet-poor plasma and determined the thrombin dynamics of TG data measured with the ST Genesia, which is a new and fully automated TG assay. In addition, we applied the thrombin dynamics method in platelet-rich plasma from healthy donors.

Chapter 2

In chapter 2 we investigated the influence of individual coagulation factors of the prothrombinase complex and natural thrombin inhibitors on prothrombin conversion and thrombin inactivation using TG and thrombin dynamics analysis. We showed that prothrombin conversion is influenced not only by the procoagulant clotting factors prothrombin and factor (F)X, but also by antithrombin, a natural thrombin inhibitor. Thrombin inactivation is mainly dependent on the thrombin inhibitors and on fibrinogen. We also determined reference values for thrombin dynamics that provide guidance for future clinical studies. We found that in a normal population, men have lower thrombin activity than women. In addition, the use of oral contraceptives (OC) significantly increases prothrombin conversion and thrombin inactivation, which may explain the increased risk of thrombosis in women taking OC. In addition, we showed that prothrombin conversion and thrombin inactivation were significantly lower in hemophilia A patients compared to healthy controls.

Chapter 3

In chapter 3, we collected plasma samples from 55 healthy donors and 189 HIV patients, of whom 96 were treated with abacavir, 93 with tenofovir disoproxil fumarate (TDF) and 19 with other drugs. TG was measured and thrombin dynamics was performed to quantify prothrombin conversion and thrombin inactivation. Patients treated with abacavir had an increased prothrombin conversion in combination with an increased thrombin inactivation, which led to a rebalanced TG. The higher prothrombin conversion in the abacavir-treated patients revealed a pro-clotting mechanism, which could explain the higher risk of thrombosis in these patients. On the contrary, patients treated with TDF had an increased thrombin inactivation, but an unchanged prothrombin conversion, which resulted in a lower TG. This could, among other things, explain the fact that these patients have a lower risk of developing thrombosis compared to abacavir-treated HIV patients.

Chapter 4

In chapter 4 we measured TG with the ST Genesia in platelet-poor plasma. With this new device, we determined the reference values in 112 healthy donors for all parameters of the thrombin dynamics method. For this study, we used the three ST Genesia reagent kits, namely STG-BleedScreen, STG-ThromboScreen and STG-DrugScreen. These reference values can be used for future research and in clinical studies. In addition, our data showed that the parameters of thrombin dynamics did not differ between men and women that did not take OC. Remarkably, the use of OC increased almost all TG and thrombin dynamics parameters.

Chapter 5

In chapter 5 we performed thrombin dynamics analysis in platelet-rich plasma and studied the effect of the difference in platelet count on TG and thrombin dynamics parameters in 117 healthy individuals. We have shown that increasing the platelet count mainly affects the rate of prothrombin conversion and TG, rather than affecting the total amount of thrombin formed. In addition, we determined the reference values of TG and thrombin dynamics in platelet-rich plasma of these healthy subjects.

Conclusion

In this thesis we have further explored the clinical relevance and biological modulators of

the thrombin dynamics analysis. In addition, we determined the reference values in healthy donors for the analysis of thrombin dynamics in platelet-poor and platelet-rich plasma. Finally, we also investigated the effect of platelets on the individual parameters of the thrombin dynamics method.

Chapter 7

NEDERLANDS SAMENVATTING

Nederlands samenvatting

De trombinegeneratie (TG) assay is een test om de stolling te meten en wordt wereldwijd vooral gebruikt in wetenschappelijk onderzoek, maar ook in de kliniek om bijvoorbeeld patiënten te monitoren. Het voordeel van het meten van TG is dat je met deze assay verschillende stollingsafwijkingen kan detecteren, alsook het risico op het verkrijgen van een bloeding of een trombose. Dit is in contrast met de conventionele testen, zoals de stollingstijden, die nauwelijks kunnen verkorten en bijgevolg een verhoogd risico op trombose niet kunnen detecteren. Sommige specifieke afwijkingen kunnen echter niet worden vastgesteld door TG alleen. Kremers et al. ontwikkelde een nieuwe methode om de belangrijkste pro- en antistollingsprocessen die de TG onderliggen meer in detail te kunnen onderzoeken door een computationele analyse van de dynamiek van de vorming van trombine. Door de trombinedynamiek-methode toe te passen verkrijgt men het verloop van de conversie van protrombine tot trombine, alsook van de trombine-inactivatie, die beiden de TG bepalen. De trombinedynamiek-analyse werd eerder beschreven voor het analyseren van TG gemeten in bloedplaatjesarm plasma, en verschillende klinische onderzoeken hebben het nut ervan aangetoond voor het verklaren van veranderde TG-profielen bij verschillende pathologieën. In dit proefschrift hebben we de klinische relevantie van de trombinedynamiek in bloedplaatjesarm plasma verder onderzocht en de trombinedynamiek bepaald van TG data die gemeten zijn met de ST Genesia. De ST Genesia is een nieuwe TG assay die volledig geautomatiseerd is. Daarenboven hebben we de trombinedynamiek methode toegepast in bloedplaatjesrijk plasma van een gezonde donoren.

HOOFDSTUK 2

In hoofdstuk 2 hebben we de invloed onderzocht van de individuele stollingsfactoren van het protrombinasecomplex en de natuurlijke trombineremmers op de protrombineconversie en de trombine-inactivatie met behulp van de TG en de trombinedynamiekanalyse. We toonden aan dat de protrombineconversie niet alleen beïnvloed wordt door de procoagulante stolfactoren protrombine en factor X, maar ook door antitrombine, een

natuurlijke trombineremmer. De trombine-inactivatie is voornamelijk afhankelijk van de trombineremmers en van fibrinogeen. We hebben ook referentiewaarden bepaald voor de trombinedynamiek, die richtwaarden bieden voor verdere klinische en wetenschappelijke studies. We hebben vastgesteld dat in een normale populatie mannen een lagere trombineactiviteit hebben dan vrouwen. Daarenboven verhoogt het gebruik van de pil de protrombineconversie en de trombine-inactivatie aanzienlijk, wat een verklaring kan zijn voor de verhoogde kans op trombose bij vrouwen die de pil slikken. Bovendien toonden we aan dat de protrombineconversie en de trombine-inactivatie significant lager waren bij hemofilie A-patiënten in vergelijking met gezonde controles.

HOOFDSTUK 3

In hoofdstuk 3 verzamelden we plasmastalen van 55 gezonde donoren en 189 HIV-patiënten, van wie 96 werden behandeld met abacavir, 93 met tenofovir disoproxil fumarate (TDF) en 19 met andere geneesmiddelen. De TG werd gemeten en de trombinedynamiek werd geanalyseerd om de protrombineconversie en de trombine-inactivatie te kwantificeren. Patiënten die werden behandeld met abacavir hadden een verhoogde protrombineconversie in combinatie met een verhoogde trombine-inactivatie, wat leidde tot een nieuwe balans van de TG. De hogere protrombineconversie bij de met abacavir-behandelde patiënten bracht een pro-stollingsmechanisme aan het licht, wat het hoger risico op trombose bij deze patiënten zou kunnen verklaren. Integendeel, patiënten die met TDF werden behandeld, hadden een verhoogde trombine-inactivatie, maar een onveranderde protrombineconversie, wat in een lagere TG resulteerde. Dit zou onder andere een verklaring kunnen zijn voor het feit dat deze patiënten een lager risico hebben op het verkrijgen van een trombose dan abacavir-behandelde HIV patiënten.

HOOFDSTUK 4

In hoofdstuk 4 is de TG gemeten met een ST Genesia in bloedplaatjesarm plasma. We

hebben met dit nieuwe apparaat referentiewaarden bepaald bij 112 gezonde donoren voor alle parameters van de trombinedynamiek methode. Voor de studie hebben we drie ST Genesia-reagentia gebruikt, namelijk de STG-BleedScreen, STG-ThromboScreen en STG-DrugScreen. De bepaalde referentiewaarden kunnen worden gebruikt voor toekomstig klinisch en wetenschappelijk onderzoek. Onze data toonden aan dat de parameters van de trombinedynamiek niet verschilden tussen mannen en vrouwen zonder anticonceptie. Opmerkelijk was dat het gebruik van de pil relateerde met een verhoging van bijna alle parameters van de TG en de trombinedynamiek.

HOOFDSTUK 5

In hoofdstuk 5 hebben we de trombinedynamiek analyse uitgevoerd in bloedplaatjesrijk plasma, en daaarmee het effect bestudeerd van het verschil in het aantal bloedplaatjes op de TG en de parameters van de trombinedynamiek bij 117 gezonde individuen. We hebben aangetoond dat het verhogen van het aantal bloedplaatjes vooral de snelheid van de protrombineconversie en de TG beïnvloedt, in plaats van de totale hoeveelheid gevormde trombine te affecteren. Bovendien hebben we de referentiewaarden van de TG en de trombinedynamiek bepaald in het bloedplaatjesrijk plasma van deze gezonde personen.

CONCLUSIE

Tot slot, in dit proefschrift zijn we dieper ingegaan op de klinische relevantie en de biologische modulatoren van de trombinedynamiek analyse. Daarnaast hebben we de referentiewaarden bepaald bij gezonde donoren voor de analyse van de trombinedynamiek in bloedplaatjesarm en -rijk plasma. Tenslotte hebben we ook het effect van de bloedplaatjes op de individuele parameters van de trombinedynamiek onderzocht.

APPENDIX I

IMPACT

SOCIETAL RELEVANCE

Cardiovascular diseases (CVDs) are the leading cause of death worldwide [1]. In 2019, an estimated 17.9 million people died from CVDs, representing 32% of all global deaths, of which 85% were caused by either a heart attack or a stroke. A significant part of CVDs can be prevented by addressing behavioral risk factors such as tobacco use, unhealthy diet, obesity, physical inactivity, and harmful use of alcohol [2]. Changes in coagulation either primary or as a consequence of behavioral risk factors play a major role in this process. Therefore, it is important to detect CVDs as early as possible to enable lifestyle changes or prophylactic treatment with medication to reduce the risk of thrombotic events [3].

OPPORTUNITIES OF THROMBIN GENERATION AND THROMBIN DYNAMICS

Thrombosis is a major complication of CVDs, which can result in myocardial infarction, acute ischemic stroke, or venous thromboembolism (VTE) [4,5]. Therefore, a suitable diagnostic test that can accurately predict the risk of thrombosis and assess the status of the hemostatic system is important in the reduction of cardiovascular events [6,7]. Conventional coagulation assays, such as the prothrombin time and the activated partial thromboplastin time were developed to detect a bleeding tendency, and are not applicable for the prediction of an increased risk for thrombosis [8,9]. The thrombin generation (TG) assay is a tool that gives a comprehensive insight into the coagulation capacity of an individual, and can be used to predict the risk of bleeding or thrombosis [10,11]. Furthermore, TG can be used to monitor patients on anticoagulant and anti-platelet treatment [12,13]. However, due to the global nature of the TG test, it is difficult to pinpoint specific defects by TG alone [9,14,15]. Therefore, by analyzing the pro- and anti-coagulant processes in thrombin dynamics, one can extract prothrombin conversion and thrombin inactivation from the underlying TG that could help clinicians to make treatment decisions for patients [16,17].

We wanted to demonstrate the usefulness of the thrombin dynamics analysis by illustrating it with a clinical example. Patients with an HIV infection are treated with a combination

anti-retroviral therapy (cART) that effectively suppresses the replication of the HIV-virus, thereby greatly reducing morbidity and mortality. The use of cART is associated with a persistently activated coagulation system and an increased risk of CVDs and VTE. Abacavir is one of the nucleoside reverse-transcriptase inhibitors and has been reported to increase the incidence of myocardial infarction [18,19]. The biological mechanism underlying the observed CVD risk associated with abacavir use remains unclear, and possible mechanisms include an abacavir-induced vascular wall inflammation, impairment of endothelial function, and platelet hyper-reactivity. In Chapter 3, we found that the higher prothrombin conversion in abacavir-treated patients contributes to the prothrombotic phenotype, which explains the higher number of thrombotic events observed in these patients. Investigating thrombin dynamics analysis of the underlying TG can be helpful to study the mechanism of cART-related thrombotic risk.

INTRODUCTION INTO THE CLINIC

Thrombin dynamics based on TG data generated by the semi-automated Calibrated Automated Thrombinography (CAT) method has been studied in multiple clinical settings over the past years to analyze the balance between pro- and anticoagulant mechanisms in patients with e.g. liver disease and hemophilia A. It also has been shown to be useful for *in silico* experimentation to investigate how differences in coagulation factor levels affect TG and thrombin dynamics parameters [16,20,21].

Since the TG test has been fully automated on the ST Genesia analyzer, it can also be performed in clinical laboratories. Therefore, we can also investigate thrombin dynamics using TG data obtained with the ST Genesia to detect discrepancies between healthy subjects and patient populations [22,23]. In Chapter 4, we used the ST Genesia to measure TG in plasma of 112 healthy donors and used the data as an input for thrombin dynamics analysis. The analyzed thrombin dynamics data can be used as reference values by other laboratories, that are able to use these data to provide guidance to differentiate between clinically ‘normal’ and ‘abnormal’ thrombin dynamics parameter values. Therefore, the

introduction of the ST Genesia into the clinic is an opportunity to use the thrombin dynamics analysis in specified clinical settings.

SCIENTIFIC IMPACT

Thrombin is the central enzyme of the coagulation system. The formation of thrombin is regulated by platelets, coagulation factors and thrombin inhibitors [11,24]. The study described in Chapter 2 further clarifies the role of FII, FV, FX and antithrombin in the regulation of the coagulation system by measuring prothrombin conversion and thrombin inactivation [25].

Studies of the conversion of prothrombin to thrombin yielded important information concerning clotting abnormalities in various hemorrhagic disorders. On the other side, studies of thrombin inactivation provide an opportunity to study anticoagulant deficiency and therapy in hemostatic disorders, such as targeting the anticoagulant pathway in haemophilia. Chapter 5 gives insight into the effect of the platelet count on plasmatic coagulability and shows that the platelet count can influence prothrombin conversion rather than the inactivation of thrombin [26]. Thrombin dynamics in platelet rich plasma could be a novel tool to study the function of platelets in coagulation on a clinical level. Previous studies have shown that thrombocytopenia, a condition in which a low platelet count can cause mild or severe bleeding events, is associated with lower TG as well [24]. In the future, platelet rich plasma-thrombin dynamics could contribute to the diagnosis and management of patients suspected of suffering from platelet disorders.

CONCLUSION & PROSPECTS

In this thesis, we present novel applications of the thrombin dynamics approach, in both its original research setting, as well as in a clinical setting, using the fully automated TG analyzer ST Genesia. Our results gave us a better understanding of how coagulation factors and platelets influence prothrombin conversion, thrombin inactivation and ultimately TG. In the past, thrombin dynamics analysis had shown its applicability to study the thrombotic/hemostatic disorders in platelet poor plasma. In this thesis, we studied the

influences of thrombin dynamics more thoroughly, and we validated the method for platelet rich plasma. The latter opens new possibilities to study platelet-related disorders and clinical situations in which platelets are affected.

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APPENDIX II

中文概述

中文概述

心血管疾病是全球第一大致死疾病[1]。据世界卫生组织估计在2019年全球有1790万人死于各种类型的心血管疾病。随着全球老龄化的加剧，心血管疾病的威胁将会更加严重[2]。凝血功能的失调是导致心血管疾病的主要原因之一[3]。开发和优化新型凝血酶测试方法有可能帮助我们更加深刻的理解凝血系统在心血管疾病中的重要作用以及帮助我们在实验室和临床诊断中发挥重要的作用[4]。

凝血酶在凝血系统中发挥重要的作用，它的作用受到促凝和抗凝蛋白的调节[5-7]。它不仅负责剪切纤维蛋白原产生纤维蛋白网状结构以阻止血液流失，还能影响血小板的生理功能[8]。凝血酶生成测试 (Thrombin generation, TG) 与传统的凝血时间检测实验不同，它是一种直接检测血浆和全血中凝血酶生成潜力的新型方法。已有实验证明，TG能估计体内的凝血潜力，因而有可能很好的预测出血或血栓的风险[9-11]。尽管现有的凝血酶生成测试能对凝血酶的潜力进行全面的估计，但是它无法区分这些变化的具体来源是来自于促凝通路或者抗凝通路[12, 13]。本论文的主要研究目的是开发凝血酶生成动力学 (Thrombin dynamics) 方法以深入的探究凝血功能变化的来源,以及探究凝血酶生成动力学的临床价值。

在第二章中，我们探究了一些重要的凝血因子和抗凝蛋白对凝血酶动力学的影响，包括凝血酶原，凝血因子V，凝血因子X以及抗凝血酶对凝血酶酶动力学参数的影响。我们发现凝血酶原转化主要受到凝血酶原，凝血因子V和X以及抗凝血酶的影响，凝血酶的降解主要依赖于抗凝血酶和纤维蛋白原的浓度。我们还在122个健康人中建立了不含血小板血浆的凝血酶动力学的参考范围，提供了可参考的正常人的凝血酶动力学参数的范围。另外，我们比较了男性和服用口服避孕药以及不用口服避孕药的女性，和血友病A的病人与正常人中的凝血酶动力学的参数的差异。

在第三章中，我们探究了不同抗逆转录病毒药物对艾滋病病人的凝血酶动力学参数的影响。我们发现阿巴卡韦能够增加凝血酶原的转化，可以用来解释服用阿巴卡韦的病人中发现的高血栓风险[14-16]。另外，我们发现富马酸替诺福韦二吡呋酯能够增加凝血酶的降解，从而降低凝血酶的生成，这可能与病人体内低血栓风险有关[17, 18]。

在第四章中，我们使用全自动凝血酶生成测试仪 ST Genesia 取代半自动凝血酶生成测试仪 CAT 检测了凝血酶生成。我们使用了所有当前可用的 ST Genesia 试剂(STG-BleedScreen、STG-ThromboScreen 和 STG-DrugScreen)在 112 名健康供体中确定了所有 ST Genesia 获得的凝血酶动力学参数的参考值，数据表明全自动凝血酶生成测试可以用于凝血酶动力学研究。随着试剂中组织因子浓度升高，凝血酶原转化增加；没有服用口服避孕药的女性和男性的凝血酶动力学参数没有差异。 相反，无论是否存在血栓调节蛋白，口服避孕药的使用都会增加几乎所有的凝血酶动力学参数。这为凝血酶动力学应用于临床研究提供了数据支持。

在第五章中，我们在富含血小板血浆中探究了血小板数量对凝血酶生成和凝血酶动力学的影响。我们发现血小板的数量变化影响凝血酶原的转化，但不影响凝血酶的降解。另外，血小板主要通过影响凝血酶原的转化速率而不是凝血酶原转化的总量来调节凝血酶的生成。我们推测是由于血小板主要为凝血酶生成提供了磷脂表面，血小板数量越多，可供使用的磷脂表面越多，那么凝血酶的生成速率加快 [19, 20]。

总结和展望

我们探究了重要的凝血因子，抗凝蛋白和血小板对凝血酶动力学的影响。此外，我们探究了在口服避孕药的女性，血友病 A 的病人以及服用抗逆转录病毒药物的病人中的凝血酶动力学的差异。凝血酶动力学能够区分和定义凝血酶原转化和凝血酶降解，更加细致和深入的解释凝血功能和凝血酶生成的变化。另外，开发凝血酶动力学方法能够帮助我们了解凝血酶生成的增加或减少是由于影响凝血酶原转化或者凝血酶的降解，帮助我们了解临床上血栓或出血病人的凝血酶变化的深层原因，并且有可能帮助临床医生在疾病的治疗中制定更加准确的治疗方案。

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APPENDIX III

LIST OF PUBLICATIONS

PUBLICATIONS

Qiuting Yan, Marisa Ninivaggi, Bas de Laat, Romy de Laat-Kremers. Reference values for thrombin dynamics in platelet rich plasma. (2020) *Platelets*

Romy M.W. de Laat – Kremers, **Qiuting Yan**, Marisa Ninivaggi, Moniek de Maat, Bas de Laat. Deciphering the coagulation profile through the dynamics of thrombin activity. (2020) *Sci Rep*

Wan J, Konings J, **Yan Q**, Kelchtermans H, Kremers R, de Laat B, Roest M.. A novel assay for studying the involvement of blood cells in whole blood thrombin generation. (2020) *J Thromb Haemost*

Qiuting Yan*, Audrey Carlo*, Hugo ten Cate, Romy de Laat-Kremers, Bas de Laat, Marisa Ninivaggi. Thrombin dynamics analyzed by the ST Genesia thrombin generation assay. (Submitted)

Qiuting Yan, Wouter van der Heijden, Marisa Ninivaggi, Lisa van de Wijer, Romy de Laat-Kremers, Andre J. Van der Ven, Bas de Laat, Quirijn de Mast. Abacavir treatment in HIV patients is associated with a procoagulant thrombin generation profile. (in preparation)

Poster presentation

2020 July, International Society on Thrombosis and Haemostasis (ISTH) annual congress. ***Yan Q.*** An Elevated platelet count increases the rate of prothrombin activation, but not the total amount of thrombin formed. **Poster presentation**

2020 July, International Society on Thrombosis and Haemostasis (ISTH) annual congress. ***Yan Q.*** Measuring thrombin generation in the presence of

haemoglobin on the ST Genesia. **Poster presentation**

Other publications

Qiuting Yan, Xuechu Zhen. Activation of AMPK/mTORC1-Mediated Autophagy by Metformin Reverses Clk1 Deficiency-Sensitized Dopaminergic Neuronal Death. (2017) *Mol Pharmacol*

Qiuting Yan, et al. Enhanced proliferation and neuronal differentiation of neural stem cell with vertically surface microenvironment. (2017) *J Biomater Sci Polym Ed*

Jianguo Hu, Ying Meng, Zhanqin Zhang, **Qiuting Yan**, et al. MARCH5 RNA promotes autophagy, migration, and invasion of ovarian cancer cells. (2016) *Autophagy*

Yafei Zhao, Panpan Wang, **Qiuting Yan**. Dihydromyricetin protects against cerebral ischemia/reperfusion injury via suppressing micogila mediatednuroinflammation and activation of ERK1/2-CREB signaling pathway. (2017) *Journal of Functional Foods*

APPENDIX IV

CURRICULUM VITAE

CURRICULUM VITAE

Qiuting Yan was born on November 25th, 1991, in Huaibei city of Anhui Province, China. She finished her high school education in Huaibei. In 2010, she was admitted to West Anhui University in Luan City of Anhui Province to study Pharmaceutical Engineering. After obtaining her bachelor's degree in 2014, she became a master's student at Soochow University in Soochow of Jiangsu Province. During her master, she studied Pharmacology at the College of Pharmacy. She worked on the Clk1 gene and the molecular mechanism of Parkinson's disease.

After obtaining her master's degree in 2017, she was awarded a scholarship from the China Scholarship Council to fund her PhD study at department of Biochemistry of Maastricht University and at Synapse Research Institute, under the supervision of Prof. dr. Hugo ten Cate and Dr. Bas de Laat. During her PhD study, she worked on the thrombin dynamics method and applied this method in both fundamental and clinical studies of thrombosis and hemostasis.

APPENDIX V

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时间真快啊，转眼间在一起度过了四年宝贵的时光，这段时光将会是人生中最宝贵，最特别的一段时间。

和翠姐一起做实验的那段时间是博士阶段最轻松，最快乐的时间，师姐教我做实验，分享食物，一起去找美食，请我们去你家吃饭。希望小馒头能无忧无虑的成长，和大馒头能天天开心。在没来荷兰时，俊哥就给我了很多帮助，帮我找房子，搬家，不厌其烦解答问题；感谢这几年和俊哥一起做实验，一起学习，教我打乒乓球。33 是我见过最会生活，最贴心的妹纸，是我做饭和做甜品的启蒙老师，经常给我投食，带我去逛街。希望回国以后，还能经常一起玩。亚秋总能把自己的生活安排的丰富多彩，又会弹吉他，坚持长跑。经常叫我们去你家聚餐，带很多吃的给我们，祝你在美国的生活更加精彩，多发文章，早日回国团聚。冬梅总是那么热情，像知心大姐姐一样帮助我，和军哥一起包饺子给我们吃，祝你在国内工作一切顺利。大黄，机票定的太晚没能一起回国，祝你能早日和你老婆团聚，顺利完成学业。李莉，祝你天天开心，一切顺利。思雨，感谢你几次在深夜收留我，祝你学业顺利，早日毕业。邹金幂，张幸真，感谢请我去你家吃火锅，还当免费司机送我回家，祝你们学业顺利，天天开心。龚英，很高兴认识龚哥，请我们去你家吃火锅，祝你能找到理想的工作，开心快乐。刘华杰，和杰哥一起上英语课，一起去打折村，请我们吃自助餐，祝杰哥工作顺利。刘晓松，感谢晓松请

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