Delicate interactions between plasma factors and blood cells affect thrombin generation

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TABLE OF CONTENTS

CHAPTER 1

General introduction & outline

HEMOSTASIS

The hemostatic system is indispensable for maintaining a continuous blood flow and vessel integrity. Upon vessel damage, the hemostatic system is exposed to subendothelial substances and is consequently triggered to generate a blood clot to occlude the wound site and prevent excessive blood loss.

Hemostasis is classically divided into primary and secondary hemostasis (**Fig.1**); the former is responsible for the formation of platelet aggregate at vessel injury site and the latter, also known as the coagulation cascade[1, 2], leads to thrombin generation (TG) and ultimately a fibrin mesh. However, growing evidence has shown that platelet activation and TG are more intertwined and these lead to the concept of "cell-based hemostasis"[3].

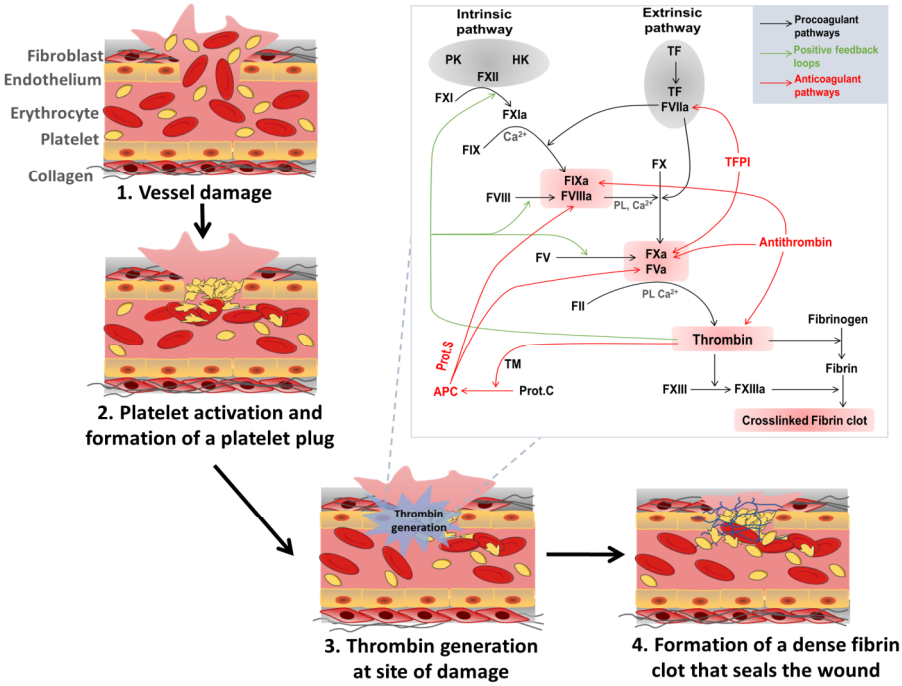

Fig.1 Overview of the classic two-stage hemostasis model. Following vessel injury, platelets adhere and form a loose aggregate upon exposure to collagen. The platelet plug is not rigid enough to seal off the wound, but activated platelets express negatively charged phospholipids (PL)[4, 5]. Thrombin generation (TG) takes place after subendothelial tissue factor (TF) is exposed to coagulation factor(F) VII and this complex consequently converts zymogens FX and FIX into FXa and FIXa, respectively. FXa, with its cofactor FVa, converts a small amount of prothrombin (FII) to thrombin. Thrombin boosts its own generation by positive feedback loops in which platelets, FXI, FVIII and FV are activated, and this results in a burst of thrombin. Fibrinogen is converted to fibrin by thrombin, and then cross-linked to a dense mesh by thrombin activated FXIIIa. TG is also confined by several anticoagulant pathways, including antithrombin, tissue factor pathway inhibitor (TFPI) and the protein C pathway. PK, prekallikrein; HK, high molecular kininogen; TM, thrombomodulin.

The new hemostasis model consists of three distinct but overlapping phases, termed the initiation, amplification, and propagation phase, respectively (**Fig.2**)[3, 4, 6]. **The initiation phase** is localized at TF-expressing surfaces (e.g. fibroblasts) where the TF-FVIIa complex is formed and generates a small amount of FXa. FXa on the cell surface then associates with FVa to form the prothrombinase complex and results in the generation of small amounts of thrombin. Any FXa dissociating from the cells following activation is rapidly inhibited by plasma inhibitors. The slowly accumulating amounts of thrombin will further activate platelets that have adhered to the site of injury, and drive FXIa, FVIIIa and FVa generation that in turn can also bind to the surface of the activated platelets (**the amplification phase**). These steps establish the key Xase and prothrombinase complexes on the activated platelet surface that drive the formation of larger quantities of thrombin during the final **propagation phase**.

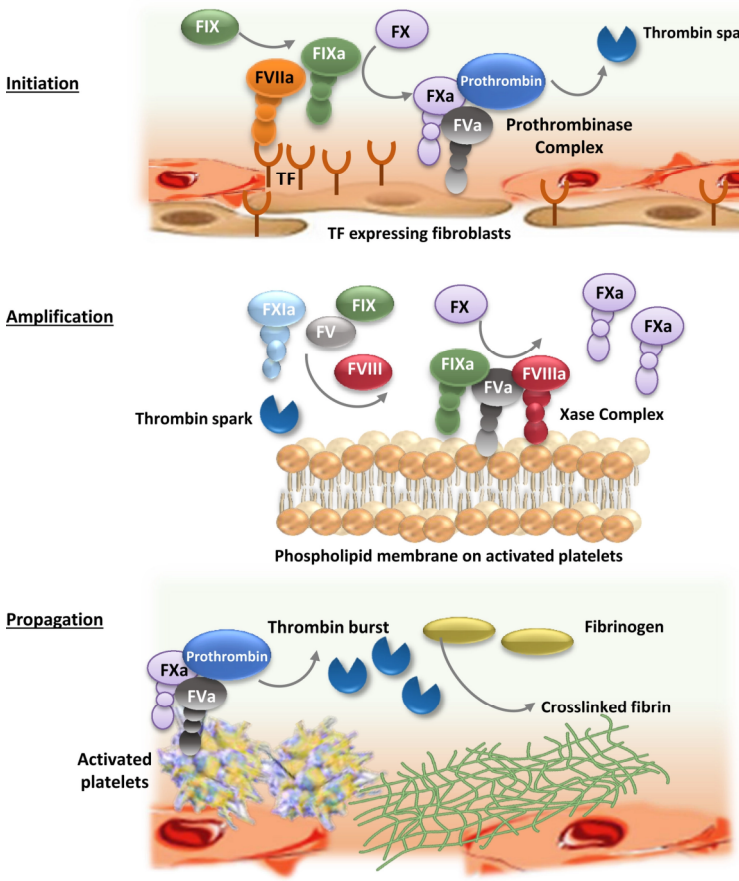

Fig.2 Cell-based coagulation. Cellular surfaces play a critical role in regulating coagulation activation in vivo. See the paragraph above for detailed explanations. Reproduced from O'Donnell et al., Br J Haematol, 2019, 186, 24–36,[6] with permission from British Society for Haematology and John Wiley & Sons Ltd.

Several natural anticoagulant proteins are present in the circulation to prevent excessive thrombin formation, including tissue factor pathway inhibitor (TFPI), antithrombin and alpha2-macroglobulin. TFPI inhibits FVIIa and FXa; whereas antithrombin and alpha2M-macroglobulin mainly target FIXa, FXa and thrombin. Thrombin also exerts anticoagulant function after binding to the endothelial receptor thrombomodulin (TM). The thrombin-TM complex activates protein C to its active form (activated protein C, APC), which degrades FVIIIa and FVa to limit further thrombin generation[7].

Normal hemostasis relies on a **balance** between pro- and anti-hemostatic pathways and their dysfunction may have life-threatening consequences. Defects in pro-hemostatic pathways can lead to uncontrolled bleeding. For example, defects in coagulation factor VIII or IX lead to hemophilia A and B, respectively. Similarly, platelet function defects (e.g. storage pool disease or receptor deficiency) are common causes of bleeding. On the other hand, malfunction of the anticoagulant pathway could lead to venous thromboembolism (VTE), as seen in individuals with deficiencies in protein C, protein S or antithrombin.

CLINICAL TESTS FOR HEMOSTATIC DISORDERS

Diagnostic laboratory tests that allow screening, diagnosis, or prediction of hemorrhagic /thrombotic risks are vital for the management of hemostatic disorders. **Table 1** provides a summary of currently available hemostatic tests [8].

The prothrombin time (PT) and the activated partial thromboplastin time (APTT), two classic clotting-based global assays devised several decades ago[9, 10], remain the most used first line screening tests of blood coagulability. PT and APTT measure the time needed for a plasma sample to form a dense fibrin clot after coagulation is triggered by extrinsic or intrinsic activators, respectively. They have been useful for monitoring treatments in severe hemophiliac patients or anticoagulation therapies (e.g. warfarin/heparin) in thrombotic patients[11]. There are several limitations of PT and APTT. In particular because fibrin forms as soon as small amounts of thrombin (~5% of all) are formed, therefore the information after the very early stages of the process of thrombin generation is not provided[12]. Furthermore, these two assays trigger coagulation with very high concentrations of trigger, which is good for achieving fast result, but this also makes the assays insensitive to moderate to mild deficiencies of coagulation factors, and is not suitable for predicting the thrombotic risks caused by malfunctions of the anticoagulant pathways[13].

Using individual coagulation factor-levels or platelet count/function to predict hemostatic potential is troublesome, due to the complexity of the hemostatic system. The advances of genomic techniques have enabled the identification of common genetic mutations such as the prothrombin G20210A mutation[14] and FV Leiden R506Q mutation[15-18] that are related with an increased venous thrombotic risk[19], but still the information they can provide is limited.

Table 1. Classification of tests for the screening and diagnosis of hemorrhagic and thrombotic disorders *

Classification	Hemorrhagic disorders	Thrombotic disorders
First-line, All clinical laboratories	Prothrombin time (PT), Activated partial thromboplastin time (APTT), Fibrinogen level, Platelet count, Platelet function screening	D-dimer, PT/international normalized ratio (INR)
Second-line, "Hub" laboratories	Mixing test, Clotting factors deficiency, Titration of inhibitors, von Willebrand disease testing, Platelet aggregation studies, Molecular testing (SNPs identification)	Deficiency of natural inhibitors, Activated protein C resistance (APCr), Lupus anticoagulants (LAC), Anticardiolipin antibodies, Molecular testing (SNPs identification)
Third-line, Reference hemostasis laboratories	Flow cytometry, Molecular testing (gene sequencing), Thromboelastography, Rotational thromboelastometry, Thrombin generation	Molecular testing (gene sequencing), Thrombin generation

**Modified from Lippi et al., J Lab Precis Med, 2018;3:67,[8] with permission from AME Publishing Company. SNP, single nucleotide polymorphism.*

GLOBAL COAGULATION ASSAYS

Recent technological advancements have led to the invention of several assays that measure the whole kinetics of thrombin/fibrin formation to provide a global assessment of the hemostatic system. Compared with traditional endpoint assays, these kinetic measurements provide more in-depth information about one's coagulation potential. Here we describe the principle of the two most studied global assays - the viscoelastic assays and the TG assays.

Viscoelastic assays

First reported in 1948 [20], Thromboelastography (TEG) utilizes mechanical rotation to detect global dynamics of clot formation, stabilization and dissolution. The two most common commercial viscoelastic testing devices are TEG® (Haemonetics®, United States) and rotational thromboelastometry (ROTEM®, Tem International GmbH, Munich, Germany). Both these tests measure the viscoelastic property of a whole blood sample after initiating with various triggers[21]. They not only provide the time of initial fibrin clot formation, but also information on platelet function and fibrinolysis. These assays have shown usefulness in reducing blood product transfer in surgeries[21], but they are too sensitive to changes in fibrinogen concentration and their ability of predicting bleeding/thrombosis risk is unclear[22].

Thrombin generation assay in plasma

The TG assay, a method to study of the potential of a given blood sample to form thrombin, is another global coagulation assay[13, 23]. The pioneering work of TG testing already emerged in

1953 by using a subsampling method[24], however, the modern fluorogenic TG assay that allows continuous and high throughput measurement was not available until 2003 when it was introduced by Hemker and colleagues[25, 26]. The commercially available assay, the Calibrated Automated Thrombography (CAT) (Stago, Gennevilliers, France), measures the transient concentrations of active thrombin over time in platelet poor plasma (PPP) or platelet rich plasma (PRP) by continuously monitoring the cleavage of a fluorogenic thrombin substrate (**Fig. 3**). The CAT assay is more user-friendly than the previous subsampling method[24] or the chromogenic method[27] and is therefore now introduced to hundreds of laboratories worldwide.

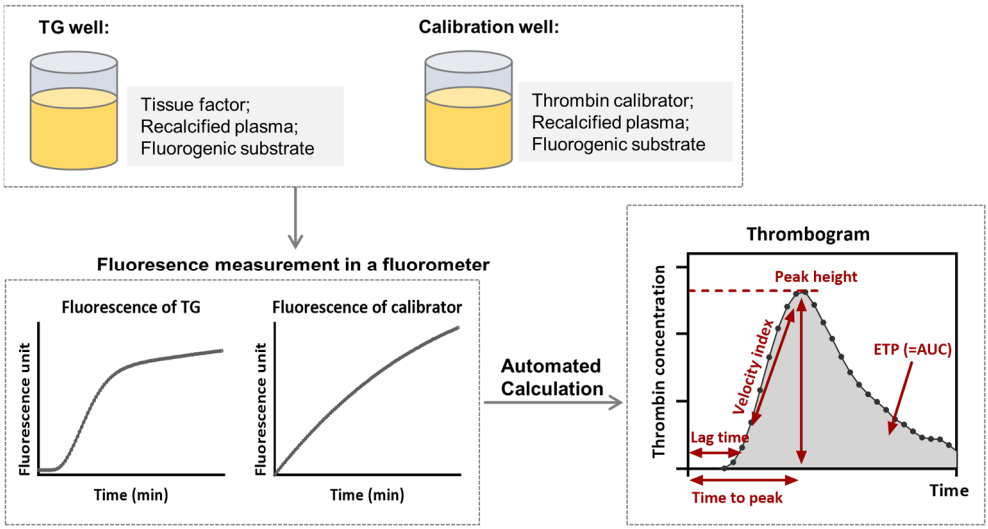

Fig.3 The setup and principle of the Calibrated Automated Thrombography (CAT) assay. This assay measures the fluorescent signals generated by the cleavage of fluorogenic substrate by thrombin after coagulation in plasma is triggered by tissue factor, as well as the signal of a parallel calibration well in which the substrate is mixed with a thrombin calibrator. A dedicated software is then used to transform the above fluorescence data into a thrombogram, which can be characterized by 5 parameters: (1) the initiation time of TG is defined as lagtime; (2) the time needed to reach maximum thrombin concentration is time to peak; (3) the highest active thrombin concentration is peak height; (4) the area under the TG curve (AUC) is called endogenous thrombin potential (ETP); (5) velocity index is calculated by dividing peak height by difference of time to peak and lagtime.

TG assays usually trigger coagulation with low amount of TF so that coagulation depends on the positive feedback loops and is also sensitive to the anticoagulant pathways. Therefore, these tests provide information on the whole process of prothrombin activation and thrombin decay, thus provide a more complete picture of the interactions between pro- and anticoagulant factors as well as their cofactor, in contrast to the PT and APTT assays that only measure the very early stages of thrombin formation[13]. Ample evidence has shown that thrombin generation assays have great usefulness in clinical laboratories, including elucidation of coagulation mechanisms, investigation of hemorrhagic coagulopathies, monitoring replacement therapy in hemophilia,

risk prediction of recurrent venous thromboembolism and monitoring of antithrombotic drugs[13, 28, 29].

TG in whole blood

To get one step closer to physiology, recently many efforts have been made to develop assays that can measure continuous TG in whole blood (WB)[30-34]. WB-TG is attractive because: (1) it includes all circulating blood cells and therefore is a straight-forward way to assess the influence of blood cells in coagulation; (2) it removes the centrifugation steps that are needed for plasma tests and thus permits faster results. Unfortunately, currently available WB-TG assays have several limitations including poor reproducibility[32], requiring complex handling[30] or can only measure one sample per time[33, 34]. Therefore, the development of novel WB-TG assays is of great interest and is a primary aim of this thesis.

INFLUENCE OF BLOOD CELLS ON COAGULATION

Cell surface-based coagulation

According to the well-accepted cell-based hemostasis model by Hoffman and Monroe[3, 4], coagulation is tightly regulated by the properties of the surface of the cell. For instance, subendothelial fibroblasts express TF and TF is able to initiate hemostasis and generate a small amount of thrombin. However, because fibroblasts lack PS exposure, the cellular surface is unable to further support the amplification of TG. Platelets, on the contrary, are well-equipped with features that makes them an ideal procoagulant surface. Once platelets are activated by subendothelial collagen or thrombin, PS is expressed on the outer leaflet of the plasma membrane to allow the binding and localization of coagulation factors that have gamma-carboxyglutamic (Gla) domain. Platelet membrane receptors, including integrin αIIbβ3 (binds fibrinogen and von Willebrand factor (vWF)) and glycoprotein (GP) Ib-IX-V (binds thrombin and vWF), also facilitate the enrichment of coagulation factors on their membrane. Furthermore, activated platelets release procoagulant proteins (FV, FXI, fibrinogen, etc.) and small molecules (e.g. polyphosphates and ADP) that augment coagulation and activate surrounding platelets. The above-mentioned characteristics of platelets support the amplification of TG from the initial formed thrombin and prepare ample reactants to allow a burst of thrombin formation during the propagation phase[3, 4].

Besides platelets, erythrocytes are also major providers of phosphatidylserine[35-37] and erythrocyte membranes trigger contact activation[38, 39]. Furthermore, leukocytes and cancer cells may be important players in cell-mediated coagulation because, under certain conditions, they express tissue factor, release procoagulant components and can induce platelet activation[40, 41].

Involvement of blood cells in coagulation disorders

Dysfunction of blood cells in some diseases maybe a critical cause of the high thrombosis risk in these disorders. For instance, tumor cells of different types of cancer are known to express TF on their outer surface, [42-44] which may explain their ability of supporting in vitro TG and

probably relates with the high risk of VTE in these patients.[45-47] The misformed erythrocytes in sickle cell disease (SCD) may also contribute to the development of VTE, as evidenced by the observation that the PPP-TG of SCD patients was comparable or lower than in healthy controls but the WB of SCD patients was able to form more thrombin-antithrombin (TAT) complexes[48]. This cellular source of VTE risk factors cannot be studied in PPP, therefore, WB-TG is a promising technique for future clinical studies to investigate the thrombosis risk in different diseases including cancer, SCD and inflammatory disorders. See **chapter 5** of this thesis for a more detailed review on the influence of blood cells on TG [49].

DISTURBED COAGULATION SYSTEM IN LIVER CIRRHOSIS

The liver is the primary organ that synthesizes coagulation proteins, therefore, patients with liver disease have complex coagulation distortions, with reduced production of most procoagulant proteins (factors II, IX, X and XI, except factor VIII) and anticoagulant factors (protein C, protein S and antithrombin)[50, 51]. Historically, bleeding was considered to be the main clinical manifestation of cirrhosis-related coagulopathy, in line with the prolongation of the traditional coagulation parameters PT and APTT[52]. However, several studies found that patients with cirrhosis are not protected but rather have a significantly increased risk of thrombotic events, particularly VTE and portal vein thrombosis[53, 54]. It is also widely accepted that many bleeding events are not caused by defective hemostasis in these patients, but rather by portal hypertension, and mechanical factors, such as vessel wall puncture during invasive procedures[50, 52]. The PT and APTT cannot give an accurate representation of the overall coagulation phenotype in cirrhosis because they are to a certain extent insensitive to the dysfunction of the anticoagulant pathway.

In contrast, TG, in particular TM-modified TG allows for a comprehensive profiling of both the pro- and anti-coagulant pathways. Tripodi et al were the first to use the TM-modified TG assay for coagulation assessment in cirrhosis and found that cirrhotic patients had similar ETP values in presence of TM (ETP^{TM+}) as controls[52, 55], which is an essential evidence for the establishment of rebalanced coagulation in cirrhosis. The same group later also determined the PRP-TG profile of these patients and showed that their ETP^{TM+} was comparable to controls if platelet count was adjusted to $100*10^9$/L, whereas the ETP^{TM+} in cirrhotic became significantly lower if the platelet number was adjusted to their respective whole blood level, suggesting a role for platelets in determining the TG capacity[56]. Considering that anemia is also very common in cirrhosis, and that erythrocytes are one of the main providers of physiological procoagulant phospholipids[37], a WB-TG assay that reflects the influence of erythrocytes on TG may provide additional information.

COAGULATION OF VIRALLY SUPPRESSED PEOPLE LIVING WITH HIV

HIV virus infection is often associated with an increased risk of venous and arterial thrombosis, which can be predicted by plasma markers of inflammation (C-reactive protein, interleukin-6) and coagulation (D-dimer)[57, 58]. The widespread use of combination antiretroviral therapy (cART) has greatly reduced AIDS-defining morbidities (deaths related to opportunistic infections) in people living with HIV (PLHIV), but many studies showed that these individuals still have increased inflammation (C-reactive protein and interleukin-6) and increased in-vivo coagulation markers (D-dimer, antithrombin-thrombin and prothrombin fragment F1+2), and are suffering catastrophic thrombotic events including myocardial infarction and stroke[58]. Furthermore, certain cART regimens that include protease inhibitor have been related with an increased risk of arterial thrombosis[59, 60]. Similarly, regimens containing Abacavir have been associated with an increased risk for arterial thrombosis in some[61, 62], but not all studies[63].

TG has been studied in PLHIV using both the in-vitro functional CAT method[64-66] and in-silico simulation methods[67, 68], but the results were inconsistent. In silico TG was shown to be lower in those on cART than those that were not on cART [67, 68]. These results were similar to another study that showed that CAT-ETP and D-dimer were lower in PLHIV when on cART than those who were not[66]. In contrary, in another study the ETP was similar between patients regardless of cART[64], although the same study showed the ETP in PLHIV was overall lower compared to uninfected controls. Interestingly, ETP was lower in PLHIV on cART who had low high sensitive C-reactive protein (hsCRP < 1mg/mL) compared with those with higher hsCRP[65], suggesting a link between inflammation and coagulation. However, the same study showed that the use of Abacavir was not associated with a different ETP or D-dimer, although the number of individuals on Abacavir was low (n=27). Overall, the TG profiles of PLHIV is still unclear, so is the effect of Abacavir use.

GENOME WIDE ASSOCIATION STUDIES TO DISCOVER THROMBOSIS-RELATED GENETIC VARIATIONS

VTE is a multifactorial disease with a strong genetic component. Family and twin studies indicate that genetics accounts for about 60% of the risk for VTE[69, 70]. Although many inherited mutations of VTE are known, including those related to antithrombin, protein C, protein S, FV Leiden and prothrombin G20210A, the majority of thrombophilic mutations remains unknown[71].

Genome wide association studies compare the genetic information (typically single-nucleotide polymorphisms [SNPs]) in relation to a particular trait (e.g. VTE) to discover new genetic variations related with the target trait. This approach has led to approximately 10,000 robust associations with diseases, quantitative traits, and genomic traits[72], including several variations related to VTE susceptibility[71, 73]. Using an intermediate phenotype may represent a more powerful approach for the identification of novel genetic risk factors of VTE because this

approach focusses to a particular disease pathway and excludes some acquired influences, thus increases the power of picking up individual genetic effects contributing to VTE[74].

TG profiles have been shown to be a useful intermediate phenotype for discovering genetic variations related with VTE[74-77]. In a proof-of-concept study by Segers et al, several known genetic variations were successfully correlated with TG and APC-modified TG parameters in a healthy population[74]. Importantly, Rocanin-Arjo et al succeeded in using the TG profile to discover a new locus, the *ORM1* gene, to be significantly associated with lag time variability and functionally proved that orosomucoid concentration was associated with impaired TG[77].

OUTLINE OF THIS THESIS

To allow a better understanding of the involvement of blood cells in thrombin generation and coagulation, we developed in **Chapter 2** a novel fluorogenic thrombin generation assay that achieved reliable measurements in whole blood. We studied the influence of the counts of platelets/erythrocytes, as well as platelet activation/inhibition on whole blood thrombin generation. The reference range of the assay was determined in 119 healthy individuals. In **Chapter 3** we optimized the performance of the Synapse prototype near patient thrombin generation assay. A new chip design was adopted to prevent fluid leakage as seen in the previous version. This assay was used to explore the whole blood thrombin generation potential of a group of cirrhotic patients for the first time. In **Chapter 4** we studied the mechanism behind the weak inhibitory effect of soluble thrombomodulin in whole blood thrombin generation. In particular, the inhibitory effect of soluble thrombomodulin was directly compared between whole blood and autologous PRP, as well as with and without supplementation of blood cells in PPP. In **Chapter 5** the added value of blood cells on thrombin generation was reviewed. **Chapter 6** describes a study that compared the plasmatic coagulability of HIV-infected individuals on antiretroviral treatment with healthy controls. The effect of different antiretroviral drugs on coagulability was also explored. Lastly, in **Chapter 7** we conducted a genome wide association study to explore the genetic determinants of (thrombomodulin-modified) thrombin generation. A summary and general discussion of the research projects are shown in **Chapter 8**.

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CHAPTER 2

A novel assay for studying the involvement of blood cells in whole blood thrombin generation

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ABSTRACT

Background: Fluorogenic thrombin generation (TG) assays are commonly used to determine global coagulation phenotype in plasma. Whole blood (WB)-TG assays reach one step closer to physiology by involving the intrinsic blood cells but erythrocytes cause variable quenching of the fluorescence signals, hampering its routine application.

Objective: To develop a new assay for continuous WB-TG measurement.

Methods: In the new WB-TG assay, the erythrocyte-caused distortion of signal was solved by continuously mixing the sample during the measurement. The assay was validated by evaluating the reproducibility and comparing with the paper-based WB-TG assay (Ninivaggi et al., 2012). Reconstituted human blood and WB from 119 healthy donors was tested to explore the influences of hematocrit and platelet count on TG.

Results: This novel WB-TG assay showed good reproducibility, while being less affected by contact activation compared with the previous paper-based assay. Reconstitution experiments showed that the Lagtime of TG was shortened by the addition of platelets but not erythrocytes. Increasing hematocrit strongly augmented the Peak thrombin, even in the presence of high platelet counts. The Lagtime and Peak of WB-TG of 119 healthy donors were positively related to erythrocyte count after adjusting for age, sex and oral contraceptive use with multiple linear regression analyses. The reference range and inter-individual variation of WB-TG were determined in the healthy cohort.

Conclusions: A novel WB-TG assay was developed, which is a straight-forward tool to measure the involvement of platelets and erythrocytes in TG and may assist the research of blood cells-associated coagulation disorders.

Keywords: blood cells; blood coagulation disorders; platelet activation; erythrocytes; thrombin.

INTRODUCTION

There is increasing awareness that thrombin generation (TG) provides a global coagulation profile of the complex interplay between pro- and anticoagulant drivers, whereas conventional clotting time tests only provide limited information about the initiation of coagulation [1-3]. Modern TG assays, such as the Calibrated Automated Thrombinography (CAT), allow continuous and high-throughput TG measurements in plasma samples by using a thrombin-specific fluorogenic substrate [4, 5]. TG assays have shown great potential in elucidating the mechanisms behind coagulation disorders, predicting bleeding/thrombotic risk and managing hemophilia/thrombosis treatment [2, 3].

Although TG assays give more comprehensive information about coagulation compared with conventional clotting time assays, they still have some non-physiological factors, including the source of procoagulant surfaces. *In vivo* TG requires physiological phospholipids (PL), which is regulated by the interplay between coagulation factors and blood cells [6, 7]. Currently, TG is often tested in platelet poor plasma (PPP-TG), with artificial PL supplemented at saturation concentration to serve as surface for the coagulation factors. TG tested in platelet rich plasma (PRP-TG) reflects the influence of platelets [8], however, it does not give insights about the impacts of erythrocytes and leukocytes. Abnormal functions of blood cells have been suggested to contribute to coagulation disorders [8]. Importantly, both abnormally high hematocrit and abnormal function of erythrocytes are associated with an increased thrombosis risk [9]. Therefore, measuring TG in whole blood (WB) is more physiologically relevant than in plasma, especially when studying coagulation disorders that are likely blood cell-originated. Additionally, unlike plasma-TG assays, WB-TG does not require centrifugation to prepare plasma, thus avoids many pre-analytical variations and allows the development of point-of-care applications.

Several methods have been published for WB-TG measurements, however most of them are too time-consuming because these methods either rely on tedious sampling at interval time-points [10-12] or can only measure one sample per run [13]. Fluorogenic assays on a microplate reader can allow continuous and high throughput WB-TG measurements, however the hemoglobin in erythrocytes severely quenches fluorescence signals and this quenching effect varies over time as a result of erythrocyte sedimentation between reading rounds. For example, in a typical CAT setting the interval time between two reading rounds is 20 seconds, which is often longer than the time needed for an Ascent Fluorometer to move the assay plate in order to read the fluorescence signals in all sample wells. Consequently, erythrocyte sedimentation takes place during the idle time when the assay plate stops moving. Clot retraction induced by activated platelets may further worsen the uneven erythrocyte distribution. Two methods have been published trying to solve this problem [14, 15]. Tappenden et al. reported a method in which an orbital shake was applied to the assay plate during the idle time between readings, but this method still gave rather high variations and was not used in any further studies [15-17]. Restraining the erythrocytes in filter paper matrix avoids erythrocyte sedimentation by forming a thin layer of blood and this technique gives reproducible WB-TG results [14], but only if executed by an

experienced operator [18]. Another disadvantage of the paper-based WB-TG assay is the strong contact activation induced by the filter paper [14].

In the present study, we developed and validated a novel assay for continuous WB-TG measurement. The erythrocyte-caused distortion of fluorescence signals was solved by continuously mixing the whole blood sample during the entire course of measurement. This assay was compared with the previously published paper-based assay regarding the disturbance from contact activation. In addition, it was also used to explore the influences of hematocrit, platelet count and platelet activation on WB-TG.

MATERIALS AND METHODS

Study subjects and blood sample preparation

Our study protocol was evaluated by the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Center). Blood was collected into vacutainer tubes (with 3.2% sodium citrate; from BD Vacutainer System, New Jersey, United States) from healthy adults who gave full informed consent according to the Helsinki declaration and had not taken any anticoagulants or platelet inhibitors for at least two weeks and had no history of thrombosis or bleeding. Blood was kept at room temperature and used within 4 hours after collection. Cell counts were measured on a Coulter Counter analyzer (Beckman Coulter, Brea, California, United States).

Reconstitution of human blood

To determine the contribution of platelet count and hematocrit in the novel WB-TG assay, samples with varying platelet counts and hematocrits were prepared.

PRP was prepared by centrifugation of blood at 220g for 15 min; PPP was prepared by double centrifugation of blood at 2840g for 10 min. To vary platelet count in plasma, PRP was mixed with autologous PPP. In some experiments synthetic PL were added to the plasma, which were from Avanti Polar Lipids (Alabaster, United States) and prepared as previously described [4].

To yield a platelet pellet, PRP was centrifuged at 890g for 15 min with Prostacyclin I_2 (Sigma-Aldrich, Missouri, United States) and resuspended in autologous PPP. Erythrocytes were washed three times in Hepes buffer (10 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.1% w/v glucose and 0.1% w/v BSA, PH 7.4) and centrifuged at 330g for 10 min after the first two washing steps and at 890g for 10 min after the third washing step. Autologous PPP, platelets, and washed erythrocytes were reconstituted to obtain samples with different hematocrits and platelet counts as indicated.

Platelet activation or inhibition in WB

To study the effect of platelet activation and inhibition on TG in WB, citrated WB samples were incubated with a platelet activator (50 µg/mL Convulxin) or inhibitor (30 µg/mL Reopro, 10 µmol/L Iloprost or 36 µmol/L Cangrelor) at 37 °C for 10 min before TG was triggered at 1 pmol/L recombinant human tissue factor (TF; from Siemens Healthcare, Erlangen, Germany). The concentrations of these molecules were chosen as the concentration resulting in the maximum effect on PRP-TG [19]. Reopro was from Janssen Biologics, Leiden, The Netherlands. Iloprost, Cangrelor and Convulxin were from Sigma-Aldrich.

WB-TG measured with the novel assay

This novel WB-TG assay was performed following the procedure of Ninivaggi et al.[14] with major modifications. Citrated WB was firstly mixed with the substrate $(ZGGR)_2$-Rhodamine 110 (P_2Rho; Diagnostica Stago, Gennevilliers, France) solution. Subsequently a solution containing TF and $CaCl_2$ was added to the WB and mixed. The volume ratio of WB, substrate solution and TF-containing solution is 3:1:2. Of the resulting mixture, 65 µL was transferred into the detection wells. The final concentrations in the well were 50% WB, 0 - 5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho. Each blood sample was calibrated by replacing the TF-containing solution with α_2-macroglobulin-thrombin complex (α_2M-T, corresponding with 300 nmol/L thrombin activity) [4]. Measurements were performed at 37˚C and each condition was tested in triplicate. Fluorescence signals were recorded with a Fluoroskan Ascent microplate fluorometer (Thermolabsystems, Helsinki, Finland) with λ_{ex} = 485 nm and λ_{em} = 538 nm using Fluoroskan Ascent Software (version 2.6). For the measurement of reconstituted blood samples, an independent calibration experiment was performed for each hematocrit level as this influences the quenching of the fluorescent signal. Fluorescence data were corrected using the H-transform when necessary [14, 20].

To prevent the light distortion caused by erythrocyte sedimentation, we optimized the assay plate and the settings of plate movement in the Ascent software, so the WB sample was adequately mixed during the whole measurement course. In the optimized setting, the interval time was set as 6 seconds, and 36 wells were always measured, which ensures that the assay plate moves continuously without noticeable stop (idle time) between two rounds of reading. Different microplates were tested to find the best structure of the reaction wells, and the round-cornered 96-Well Assay Microplate from Corning (type number 2595; New York, United States) resulted in the lowest variation (Supplementary Table S1).

A dedicated preprogrammed spreadsheet template was used to calculate the WB-thrombogram parameters from the experimental fluorescence data as described before [13, 21-23]. The template is available upon request, the algorithms used in the calculation template is described in the Supplementary information. In brief, an extended Chapman-Richards growth (eCRG) equation, $F = a(1 - e^{-b \cdot t})^{c} + d(1 - e^{-f \cdot t})^{g}$ was fitted on the sigmoidal part of the fluorescence data, where F is the fluorescence intensity at time t and a, b, c, d, f, and g are the parameters that determine the shape of the fitted curve. The sigmoidal part of the fluorescence data was estimated after using a simple moving average technique to smooth out the noise within the fluorescence data

[5]. From the fitted curve, WB-TG parameters were calculated, including the Lagtime (min), Time-to-peak (min), Peak thrombin (nmol/L) and endogenous thrombin potential until the thrombin peak (ETPp; nmol•min/L). This algorithm used in the calculation template renders that the WB-TG parameters are derived objectively from the fluorescence data and are not disturbed by the subjectivity of the operator. To illustrate this, we let 3 operators to each independently do the calculation with a same set of fluorescence data, using the programmed calculation template. As shown in Supplementary Fig.S2, the TG parameters obtained were identical among 3 operators, meaning that the calculation method was free of the subjectivity of the operator.

Fluorescence signal of Rhodamine 110 fluorophore in clotting WB over time

The fluorescence signal of Rhodamine 110 fluorophore (Rho; the end product of $[ZGGR]_2$-Rho cleavage by thrombin) in clotting WB was monitored over time to assess the stability of the quenching effect of clotting WB on fluorescence signal. For this purpose, 2.5 μM Rho (Sigma-Aldrich) was either added into, or replaced, the substrate solution in WB-TG. Fluorescence signal was monitored as described above.

The paper-based WB-TG assay

The paper-based WB-TG assay was performed as originally described [14]. Fluorescence data were transformed into TG parameters using the CAT-method as originally described [5, 14].

Statistics

Statistical analyses were done using GraphPad 5.0 (Graphpad Software, San Diego, United States) and SPSS 25 (IBM, New York, United States). Data were checked for normality and are shown as median and interquartile range (IQR) or mean ± standard deviation (SD). Independent groups were compared using the Mann-Whitney test. Paired samples were compared using paired t-test or Wilcoxon matched-pairs test depending on normality. Pearson correlation was calculated between the parameters from the two WB-TG techniques. Reference intervals of the WB-TG parameters of healthy donors were calculated as 2.5^{th} to 97.5^{th} percentile according to the Clinical & Laboratory Standards Institute (CLSI) guideline [24]. Multiple linear regression analyses were performed with the ETPp, Peak thrombin or Lagtime of the 119 healthy donors as dependent variable and cell counts, age, sex and oral contraceptive (OC) use as independent variables. For the sex parameter, male and female were coded as 0 and 1 in the analyses, respectively. Effect modification of sex on OC use and cell counts were also considered in the regression analysis and the backward method was used to find the best model. *P* values below 0.05 were considered statistically significant.

RESULTS

Continuous mixing resulted in a stable light transmission in WB-TG

The efficacy of this continuous mixing method in preventing erythrocyte-caused variable quenching on fluorescence signal transmission was firstly tested in calibration experiments, in which the cleavage of the thrombin substrate P_2Rho by the calibrator was monitored in citrated WB. Fig. 1A shows the mean fluorescence tracing of 12 replicate experiments over 20 minutes. The first derivatives (i.e. calibrator slope) of the tracings had a coefficient of variation (CV) of 3.7% and showed a horizontal trend when plotted versus time, indicating stable light transmission and no disturbance from substrate consumption or inner filter effect.

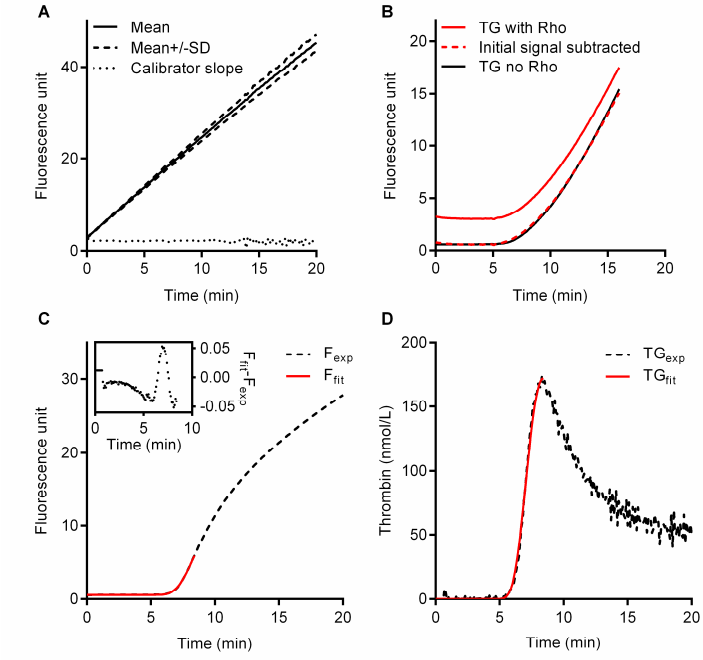

Fig. 1 The performance of this novel WB-TG assay and the CRG-based calculation method. (A), the reaction between calibrator α_2M-T (100 nmol/L thrombin activity) and substrate P_2Rho (300 µmol/L) in citrated whole blood. Mean (solid line) and mean ± standard deviation (dashed lines) of 12 replicate experiments are shown. The dotted line represents the first derivative of the average fluorescence tracing. (B), the fluorescence tracing of two parallel WB-TG experiments at 2.5 pmol/L TF in the absence (black line) or presence (red line) of exogenously added 2.5 µmol/L Rhodamine (Rho) fluorophore. The red dashed line is the residual signal after subtracting the background Rho signal from the total signal. (C), An extended Chapman-Richards growth (CRG) equation was fitted on the experimental fluorescence data from t=0 until t=Time-to-peak; the correctness of the fitting, as shown by the small difference between the fitted data (F_{fit}, red solid line) and the experimental data (F_{exp}, black dashed line) is shown in the insert. (D), the calculated thrombograms from the fitted data (TG_{fit}, red solid line) and the experimental data (TG_{exp}, black dashed line) overlap well with each other.

The performance of the mixing method in clotting WB was evaluated by comparing the fluorescence signal of two parallel TF-induced WB-TG experiments in the presence and absence of exogenously added Rho. The added Rho induced a stable background signal during the lag-phase (Fig. 1B). From t = 0 till the time when signal increased the most (i.e. Time-to-peak), the signal difference between the sample with exogenous Rho and the parallel sample without Rho addition remained relatively stable (Fig. 1B), as shown by the overlap between the curves after subtracting the Rho background signal. This was repeated in WB samples from 3 donors, and the deviations of WB-TG parameters ETPp and Peak thrombin between with and without Rho fluorophore addition were on average 2.2 ± 1.6% and 4.9 ± 3.1% (n = 4), respectively. In addition, the fluorescence tracings of Rho added to clotting blood was monitored in the absence of P_2Rho substrate, so the influence of the clot on the signal was judged independently of the amount of product generated. As shown in Supplementary Fig. S3, the signal of added Rho remained relatively stable in the first 0-10 minutes (during which TG reaches the peak), although a slight increase in signal as well as higher variation were observed in the later part (20-30 minutes) of the experiments. Collectively, these results imply that this assay setting maintained the quenching effect of erythrocytes at a stable level at least until the Thrombin-peak is reached.

Conversion from fluorescence signals to WB-TG parameters

The time dependent substrate cleavage in WB-TG (Fig. 1C and Supplementary Fig.S4) showed strong similarities with plasma-TG, however the tail part (thrombin decay) of the former had higher variation between replicates. Consequently, the estimation of the α_2M-T end level was less reliable. The first half of the WB-thrombogram (from the Lag-phase until thrombin peak), however, was highly reproducible (average CV of 18 replicate measurements was 4.6%). Therefore, we calculated the endogenous thrombin potential until the thrombin peak (i.e. ETPp) after fitting an extended CRG-curve on the sigmoidal part of the experimental fluorescence signal as described in the method section [13, 22]. Correctness of the fitting was shown by the high agreement between the experimental data and the fitted data (Fig. 1C). This calculation mode resulted in a thrombogram that correlated well with the original one (Fig. 1D and Supplementary Fig. S5).

Response of the WB-TG parameters to varying TF concentrations

The response of the WB-TG parameters to different TF concentrations is shown in Supplementary Table S2. TF dose dependently altered thrombogram parameters. The Lagtime decreased from 14.8 ± 0.6 to 2.3 ± 0.1 min when increasing TF concentrations from 0 to 5 pmol/L and a similar trend was found with the Time-to-peak. The Peak thrombin increased from 193 ± 5 nmol/L to 279 ± 1 nmol/L with increasing TF concentration but the ETPp hardly changed.

Precision of the novel WB-TG assay

The intra-assay variation of this WB-TG assay was assessed by 15 replicate measurements of a citrated WB sample at 1 pmol/L TF. As shown in Table 1, the CVs were below 6% for all TG parameters. Moreover, when this assay was measured in a population of 119 healthy volunteers

the average intra-assay CVs between triplicate measurements were 1.8%, 2.2%, 4.6% and 4.6% for the Lagtime, Time-to-peak, Peak and ETPp, respectively.

The inter-assay precision could not be determined over a time period longer than 1 day due to the storage limitation of WB. Therefore, it was determined by 18 independent experiments testing a single WB sample in triplicate resulting in CVs less than 7% for all thrombogram parameters. The inter-operator variation of this assay, determined by letting 4 operators each independently test a same WB sample, was <13% for all WB-TG parameters (Supplementary Table S3).

Table 1 Intra- and inter-assay variations of this novel WB-TG assay

	Intra-assay variation (n=15)		Inter-assay variation (n=18)	
	Mean ± SD	CV, %	Mean ± SD	CV, %
Lagtime (min)	5.7 ± 0.1	1.8	4.3 ± 0.3	6.2
Time-to-peak (min)	8.4 ± 0.2	2.0	6.7 ± 0.3	4.1
Peak (nmol/L)	173 ± 10	5.6	239 ± 14	6.0
ETPp (nmol*min/L)	260 ± 9	3.6	308 ± 20	6.5

Abbreviations: WB-TG, whole blood thrombin generation; ETPp, endogenous thrombin potential until thrombin peak.

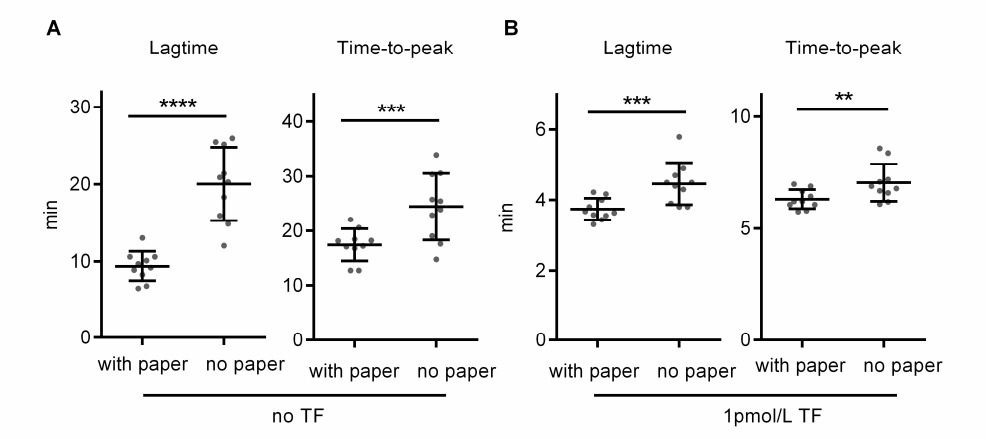

Fig. 2 Comparisons between the novel WB-TG assay and the paper-based assay. For both assays, TG was triggered at 0 (A) or 1 pmol/L TF (B) in citrated whole blood from 10 healthy volunteers. Same reagents were used in both assays. Thrombogram parameters were calculated using the CRG-assay for the novel assay without filter paper (no paper) or with the CAT-assay for the filter paper-based assay (with paper). Mean and SD are indicated as bars in the figures. Comparisons between groups were done using the paired t-test, **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

Comparison between this novel WB-TG assay and the paper-based assay

TG in WB of 10 donors was tested with the previously published paper-based assay [14] and this novel assay at 0 and 1 pmol/L TF. The Lagtime was significantly shorter in the paper-based assay, both without TF (Fig. 2A; 9.3 ± 2.0 versus 20.0 ± 4.8 min; $P < 0.0001$) and at 1 pmol/L TF (Fig. 2B; 3.7 ± 0.3 versus 4.5 ± 0.6 min; $P < 0.001$). Shorter Time-to-peak was also observed in the paper-based assay irrespective of the TF concentrations.

The TG parameters from these two assays showed significant correlations. The Pearson correlation coefficients were 0.79 (95% confidence interval [CI]: 0.32 - 0.95; $P = 0.007$), 0.75 (95% CI: 0.22 - 0.94; $P = 0.013$) and 0.82 (95% CI: 0.38 - 0.96; $P = 0.004$) for the Lagtime, Time-to-peak and ETPp, respectively. The Peak thrombin values showed weaker correlation (r = 0.5997; 95% CI: -0.05 - 0.89; $P = 0.067$).

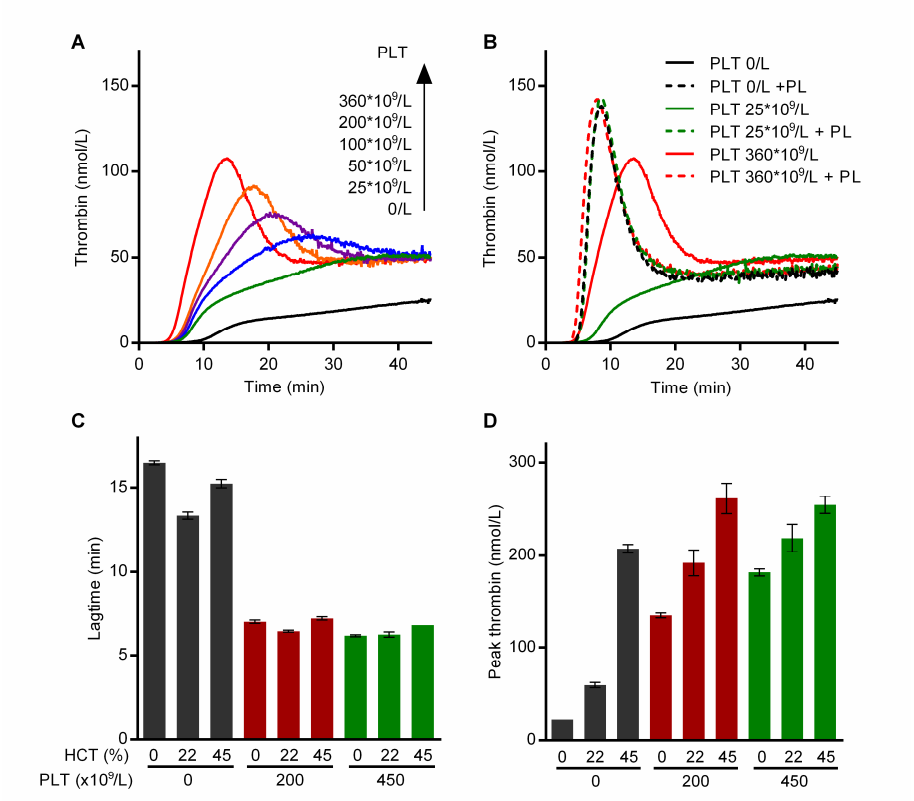

Fig. 3 TG in reconstituted blood samples as measured by the novel WB-TG assay. Human whole blood was separated into erythrocytes, platelets and PPP and then reconstituted into the indicated levels. TG was measured in reconstituted PRP with varying platelet (PLT) count in the absence (A) or presence (B) of 4 µmol/L phospholipids (PL). (C, D), TG was measured in reconstituted samples with varying platelet counts (0, 200 or 450 $\times 10^9$/L) and hematocrits (HCT; 0, 22 or 45%) as indicated in the figures. The Lagtime (C) and Peak thrombin (D) of TG are shown. Each condition was measured in triplicate.

Influences of hematocrit and platelet count on WB-TG

We determined the effect of platelet count and hematocrit on the WB-TG assay in PRP and reconstituted blood samples. Increasing the platelet count in plasma shortened the Lagtime from 11.7 min at 0 × 10^9 /L platelets to 7.4 min at 25 × 10^9 /L platelets, and further to 5.0 min at 360 × 10^9/L platelets (Fig. 3A). Gradual increments in the Thrombin-peak were found with increasing platelet counts. Addition of 4 μmol/L synthetic PL markedly augmented the Peak thrombin, even in the presence of 360*10^9/L platelets (Fig. 3B).

In reconstituted blood samples, platelets shortened the Lagtime markedly irrespective of the hematocrit level, whereas increasing the hematocrit level hardly shortened the Lagtime (Fig. 3C; Table 2). Interestingly, increasing hematocrit in the presence of 450*10^9/L platelets prolonged the Lagtime. The Peak thrombin values showed dose dependent increase with platelet count and hematocrit (Fig. 3D; Table 2). The ETPp of the reconstituted samples did not show dose dependent change.

Table 2 Thrombin generation measured in reconstituted blood using the novel WB-TG assay

Platelet (×10^9/L)	**0**	**0**	**0**	**200**	**200**	**200**	**450**	**450**	**450**
Hematocrit (%)	**0**	**22**	**45**	**0**	**22**	**45**	**0**	**22**	**45**
Lagtime (min)	16.5 ± 0.1	13.3 ± 0.2	15.2 ± 0.3	7.0 ± 0.1	6.4 ± 0.1	7.2 ± 0.1	6.2 ± 0.1	6.3 ± 0.2	6.8 ± 0
TTP (min)	NA	NA	18.5 ± 0.2	12.2 ± 0.2	10.9 ± 0.3	10.0 ± 0.1	10.0 ± 0.2	10.2 ± 0.2	9.4 ± 0.1
Peak (nmol/L)	22.3 ± 0.1	59.7 ± 2.7	207.5 ± 4.6	134.7 ± 2.6	185.6 ± 13.6	261.3 ± 16.4	181.8 ± 3.9	211.5 ± 14.2	254.3 ± 9.1
ETPp (nmol*min /L)	NA	NA	363.9 ± 18.2	390.8 ± 5.2	450.4 ± 12.0	381.3 ± 39	380.9 ± 13	446.0 ± 34.1	346.1 ± 16.1

Note: TG in 2 groups (platelet count = 0 and hematocrit = 0 or 22%) did not reach Peak thrombin level even after 50 min, consequently the ETPp could not be calculated, therefore the thrombin concentration at t = 50 min was registered as Peak thrombin value. Each condition was measured in triplicate. Data are expressed as mean ± SD (n=3). Abbreviations: WB-TG, whole blood thrombin generation; ETPp, endogenous thrombin potential until thrombin peak; NA, not available.

Influence of platelet activation/inhibition on WB-TG

To study the influence of platelet on WB-TG in more detail, different platelet agonists and antagonists were tested. As shown in Supplementary Fig. S6, Convulxin, a platelet agonist that induces phosphatidylserine exposure, shortened the Lagtime by 19.9 ± 3.7% (n = 5), but did not affect the ETPp and Peak thrombin. Addition of Reopro (inhibitor of platelet integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$) prolonged the Lagtime by 15.8 ± 8.5 % (n=5) and reduced the Peak thrombin by 17.1 ± 11.7%, but hardly affected the ETPp. The effect of Reopro was further confirmed in WB samples from 88 healthy donors in which Reopro hardly changed the ETPp (P = 0.06) but prolonged the Lagtime by 24.9% and reduced the Peak thrombin by 22% (P < 0.0001 for both)

(Supplementary Fig. S7). The addition of Iloprost and Cangrelor showed weaker inhibitory effect on WB-TG compared with Reopro.

WB-TG tested in a healthy population

Citrated WB samples from 119 healthy adult volunteers were tested to study the characteristics of WB-TG in a normal population. The reference intervals and inter-individual CVs of the WB-thrombogram parameters were determined and are shown in Table 3 together with the cell counts. Compared to WB-TG triggered at 1 pmol/L TF, similar ETPp was seen when triggered at 2.5 pmol/L TF ($P = 0.88$), along with significantly higher Peak thrombin, shorter Lagtime and Time-to-peak (Table 3; $P < 0.0001$ for all).

The volunteers consisted of 60 females and 59 males. Females had significantly higher platelet counts (median [IQR], 282.8 [164.1 - 417.8] versus 247.8 [149.4 - 372.8] $\times 10^9$/L, $P < 0.01$) and lower erythrocyte counts (4.7 [3.9 - 5.9] versus 5.1 [4.5 - 6.3] $\times 10^{12}$/L, $P < 0.0001$) than males, while having similar age, white blood cell count and mean platelet volume. Significantly shorter Lagtime and higher ETPp were observed in females than in males ($P < 0.01$ for both) (Fig.4), but the Thrombin-peak was comparable.

Age correlated with Lagtime (Spearman r = -0.212, $P < 0.05$), Peak thrombin (r = 0.215, $P < 0.05$) and ETPp (r = 0.218; $P < 0.05$). Multiple linear regression analyses were performed to explore the effect of blood cell counts on WB-TG. After adjusting for age, gender and OC use, erythrocyte count significantly impacted the Lagtime (standardized Beta = 0.188, $P = 0.034$) and Thrombin-peak (standardized Beta = 0.205, $P = 0.015$) (Supplementary Table S4). The effect of platelet count on the ETPp was borderline significant (standardized Beta = 0.159, $P = 0.065$).

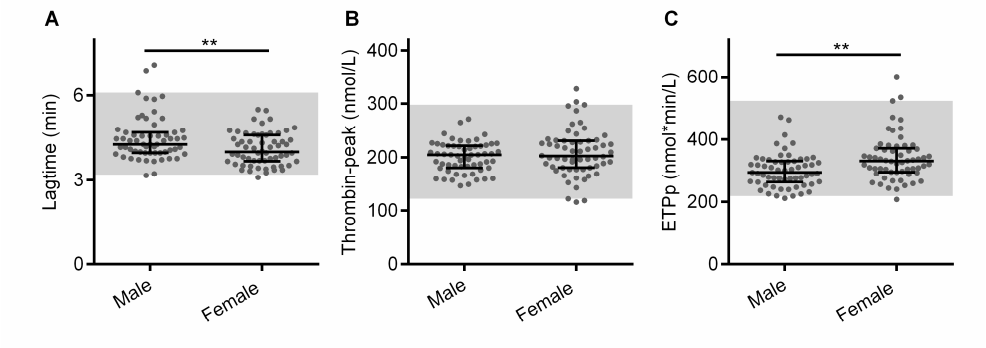

Fig. 4 The WB-TG parameters of males and females in 119 healthy volunteers. TG was triggered at 1 pmol/L TF in citrated WB samples. WB-TG parameters Lagtime (A), Thrombin-peak (B) and ETPp (C) are shown for the different groups, including males (n=59) and females (n = 60). Medians and interquartile ranges are indicated as bars; the gray areas represent the reference intervals of the total population (2.5th percentile to 97.5th percentile). Statistical significance was determined using the Mann-Whitney U-test, **: $P < 0.01$.

Table 3 Inter-individual variations and reference intervals of the WB-TG parameters

	n	%CV	Median	Reference Intervals (2.5th to 97.5th percentile)
Female, number (%)	60 (50.4%)	-	-	-
Age	119	-	31.0	20.0 - 63.0
Blood counts				
White blood cell ($\times 10^9$/L)	119	23.5%	5.8	4.1 - 8.8
Erythrocyte ($\times 10^{12}$/L)	119	8.9%	4.9	4.1 - 6.0
Hemoglobin (mmol/L)	119	8.7%	8.8	7.0 - 10.4
Hematocrit (%)	119	8.1%	42.9	35.4 - 48.3
Platelet ($\times 10^9$/L)	119	20.9%	263.3	151.1 - 397.8
Mean platelet volume (fL)	119	11.1%	7.5	6.0 - 9.8
WB-TG at 1 pmol/L TF				
Lagtime (min)	119	16.6%	4.2	3.2 - 6.1
Time-to-peak (min)	119	14.8%	7.0	5.9 - 10.6
Peak (nmol/L)	119	18.0%	203.2	122.5 - 297.7
ETPp (nmol*min /L)	119	21.1%	315.6	218.9 - 522.9
WB-TG at 2.5 pmol/L TF				
Lagtime (min)	119	15.5%	2.8	2.1 - 4.1
Time-to-peak (min)	119	12.3%	5.3	4.5 - 7.1
Peak (nmol/L)	119	15.2%	230.7	161.3 - 316.9
ETPp (nmol*min /L)	119	19.8%	307.6	222.6 - 489.7

Abbreviations: WB-TG, whole blood thrombin generation; TF, tissue factor; ETPp, endogenous thrombin potential until the Thrombin-peak.

DISCUSSION

Fluorogenic plasma-TG assays are commonly used to model the global coagulation phenotype because these assays provide an efficient tool for assessing the complex interplay between pro- and anti-coagulant factors [2, 4, 25]. WB-TG is one step closer to physiology because it also includes the influence of the circulating blood cells on TG. The erythrocytes in WB can cause distortion of the fluorescence signal transmission, as a result of erythrocyte sedimentation during measurements. Previous reported fluorogenic methods for WB-TG measurement suffered from high imprecision [15] or strong interference of contact activation [14], hampering the routine applications of WB-TG in routine applications.

In this study we developed a novel fluorogenic assay for continuous WB-TG measurement. Stable light transmission during WB-TG was achieved by continuous mixing of the assay plate during the entire measurement course, using a microtiter plate with round-cornered wells. We

obtained reproducible fluorescence signals in both the calibration and TG experiments, and we showed that erythrocyte dependent light distortion was adequately controlled in clotting blood, because exogenously added rhodamine gave a stable fluorescence signal during a whole WB-TG course.

Although the ascending part of the WB-TG curve was stable and reproducible, the descending part of a WB-TG curve had higher variation between measurements. Therefore, we used a CRG-based calculation method to calculate the endogenous thrombin potential until the Thrombin-peak (termed ETPp). Similar to previous studies [13, 22], we found that the WB-TG parameters from the CRG-based calculation method correlates well with that from the classic CAT-method (Spearman $r > 0.95$ for all TG parameters). The Thrombin-peak calculated by the CRG-method is systematically higher than that by the regular CAT-method, because no correction is foreseen for the α_2M-T that still cleaves the substrate [13]. The advantage of the CRG-based calculation is that the measurement time is significantly shorter (from 50 minutes to 20 minutes), although it may fail to give correct calculation for certain samples (e.g. samples with extremely slow TG or if the shape of the TG curve deviates from normal as is observed in the presence of direct FXa inhibitors). Of note, the mixing technique described here is optimized for the Fluoroskan Ascent fluorometer, therefore the user of the plasma-CAT assay can readily adopt this new assay, but additional optimization will be needed for other fluorometers.

We tested the reproducibility of our novel WB-TG assay and showed that the intra- and inter-assay variations of all parameters were below 7%. We also compared our novel WB-TG assay with the paper-based WB-TG assay. The paper-based assay had a shorter Lagtime (2.15 fold, on average) than our novel WB-TG when TG was not triggered with TF or another initiator of coagulation. This difference indicates that our novel WB-TG assay is less disturbed by contact activation than the paper-based assay [14, 18]. Although the use of CTI in the paper-based assay can reduce the effect of contact activation, this will increase the cost and increase the number of handling steps of the assay [14]. Another advantage of our novel WB-TG is that there is no need for preparation of paper disks or addition of oil, making it less laborious and less dependent on the skills of the operator.

The inter-individual variations of the WB-TG parameters in a group of 119 healthy volunteers was between 12% to 22%, which is comparable to the values previously reported in PPP, PRP and WB [26]. Previously identified pro-thrombotic risk factors, i.e. higher age and OC use [27, 28], were confirmed by our novel WB-TG assay; higher age and OC use were associated with shorter Lagtime, higher ETPp and Peak thrombin. In agreement with previous observations [26], females have a shorter Lagtime and higher ETPp than males. The reference ranges of the WB-TG parameters were determined, to pave the way for further studies.

TG in WB depends on a complex interplay between coagulation factors and blood cells [6, 7]. In a widely accepted cell-based model of hemostasis, TG is initiated by factor (F)VIIa binding to surface-bound TF, leading to the activation of trace amounts of FIX, FX and, subsequently, thrombin. Low concentrations of thrombin activate platelets and cofactors FV and FVIII, which form a complex with FXa and FIXa, respectively to increase the efficiency of thrombin

formation (i.e. propagation) on phosphatidylserine-positive surfaces [6, 7]. Platelets are equipped to create a pro-coagulant environment, *via* secretion of coagulation (co)factors [6], expression of membrane receptors and phosphatidylserine exposure to form the surface for the coagulation cascade [7]. The effects of platelet count and function on the onset and velocity of *ex vivo* TG was previously characterized with a PRP-TG assay [4, 19], and was also confirmed in our WB-TG assay.

The impact of erythrocyte on TG and coagulation is less well established and has gained interest [10, 11, 29, 30]. Using our novel WB-TG assay, we studied the influence of erythrocytes and platelets on TG in detail with reconstituted blood samples and in WB from 119 healthy donors. We found that the fast onset of TG (i.e. a short Lagtime) relied on the presence of platelets and was also affected by the activation or inhibition state of platelets. Conversely, high erythrocyte numbers prolonged the Lagtime, while erythrocyte count dose dependently augmented the Peak thrombin of TG, which persisted in the presence of high platelet numbers. The negative impact of high erythrocyte numbers on the onset of TG could be attributed to steric hindrance, due to its large size and high abundance in WB. Although a portion (approximately 0.5%) of erythrocytes in the circulation of normal individuals exhibited PS exposure [10, 11], this portion may be too low to reverse the above-mentioned effect. Erythrocytes may augment TG in the propagation phase once they are trapped inside the clot. Besides the PS exposure and their impact on blood rheology and endothelium function, erythrocytes have also been suggested to directly affect platelet function [31]. A recent finding showed that erythrocyte-platelet interaction via the Fas ligand-receptor contact increases the phosphatidylserine (PS) exposure on both cells [32], which could potentially facilitate TG.

In healthy subjects, the normal variation in platelet count and in hematocrit seems to have minimal effects on the ETPp. This was also observed in previous studies in PRP-TG, which showed that the ETP was insensitive to platelet numbers, if the platelet numbers were higher than 35×10^9/L [4, 19]. Furthermore, our experiments studying the influences of platelet agonist and antagonists on WB-TG also showed that these molecules mainly influenced the initiation and velocity of TG but the influence on the ETPp was neglectable. Probably, the effects of platelet inhibitors in preventing venous thrombosis stem from delaying the onset of TG on platelets. However, there are several diseases in which the high thrombotic risk is likely induced by the blood cells and not by the plasma coagulation factors, including sickle cell disease and hematologic malignancies such as multiple myeloma and leukemia [33, 34]. For example, patients with sickle cell disease are recognized to have a hypercoagulable state, resulting in higher amounts of *in vitro* thrombin-antithrombin complex formation in WB than normal controls [35], however their endogenous thrombin potential (ETP) tested in plasma was lower [35] compared to normal controls, thus suggesting that blood cells may play an important role for the hypercoagulability.

In conclusion, we have developed a novel assay for continuous WB-TG measurement. This assay is a straightforward approach to measure the involvements of platelets and erythrocytes in TG and may assist the research of blood cell-associated coagulation disorders.

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Author contributions

J. Wan, J. Konings, B. de Laat & M. Roest designed the study; H. Kelchtermans & R. Kremers organized donors and collected donor characteristics; J. Wan, J. Konings & Q. Yan performed the experiments and interpreted data; J. Wan, J. Konings & M. Roest wrote the manuscript; H. Kelchtermans critically reviewed the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY INFORMATION:

Description of the CRG-based calculation method for WB-TG

The tail part of the fluorescence tracings obtained from WB-TG experiments have high variation between replicates **(**see **Supplementary Fig.S3)**, rendering the estimation of the α2M-thrombin end level less reliable. Therefore, we decided to discard the decaying part of a thrombogram (i.e. the TG curve after thrombin peak) and only calculate the TG parameters from the ascending part of a TG curve (from t=0 until thrombin peak). Previous studies have shown that TG parameters calculated with this half-gram method correlated strongly with those calculated with the classic method, and was predictive of post-operative blood loss post cardiothoracic surgery (Kelchtermans H, et al. [Thromb Haemost, 2016] and Moorlag M, et al. [JALM, 2017]). However, due to the existence of noise in the fluorescence data **(**as shown in **Supplementary Fig.S3**, a spike could arise before the real thrombin peak point**)**, it is difficult to directly identify the exact time point that correspond to the thrombin peak (i.e. time-to-peak, TTP) from an ordinary first derivative curve.

To solve this problem, we fitted a smooth extended Chapman-Richards growth (CRG)-curve on the fluorescence data. The fitting was only applied on the sigmoidal part of the experimental fluorescence data, so that the erratic tail part of the fluorescence curve does not interfere with the fitting. To estimate the sigmoidal part of the fluorescence curve, a simple moving average (SMA) technique was used to smooth out the fluctuations caused by fluorescent noise. SMA is a technique to get an overview of the trends in a data set; it calculates an average of any subset of adjacent numbers to smooth out the noise inside. The degree of SMA was identical in every calculation to guarantee objectivity.

A screen shot of the calculation template is shown in **Supplementary Fig.S1.** To recapitulate, when the fluorescence data was pasted into the calculation template, a smoothed first derivative curve of the fluorescence data was automatically generated with the SMA technique, and the peak point on this curve was automatically identified (denoted as TTP_{SMA}) with the help of the "MAX" function embedded within the Microsoft Excel. Then, a CRG curve was fitted on the fluorescence data from t = 0 to t = $TTP_{SMA.}$ The best fitting curve was found using the "SOLVER" add-in tool within the Microsoft Excel, as the one that resulted in the least squared deviation from the fluorescence data. TG parameters were subsequently obtained from the first derivative curve of this CRG-curve.

As mentioned above, the operator only needs to paste the fluorescence data and press the "SOLVER" button within the Excel-based calculation template to get the TG parameters, therefore the calculation is not influenced by the subjectivity of the operator. As a proof of the objectivity of the calculation template, we show that the TG parameters calculated by 3 independent operators from a same set of fluorescence data was highly comparable between each other **(**see **Supplementary Fig.S2)**.

Supplementary Table S1. The %CV of WB-TG tested on different types of 96-well plates [a]

	Corning type 2595	Thermo Immuno 2HB flat-bottom	Thermo Immuno 2HB round-bottom
Lagtime	3.3	NAb	3.3
Time-to-peak	2.9	NA	4.3
Peak	3.9	NA	6.7
ETPp	4.1	NA	12.7

a: Values were determined in WB-TG experiments triggered by 2.5 pM tissue factor, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho; each condition 12 replicates. b: The %CV of WB-TG on the Thermo Immuno 2HB flat-bottom plate cannot be determined because the calibration curve and TG curve were too erratic.

Supplementary Table S2 Recombinant TF dependency of the WB-TG parameters [a]

TF (pmol/L)	Lagtime (min)	Time-to-peak (min)	Peak (nmol/L)	ETPp (nmol*min /L)
0	14.8 ± 0.6	18.1 ± 0.6	193 ± 5	345 ± 17
0.5	6.7 ± 0.1	9.5 ± 0.2	216 ± 7	322 ± 9
1	4.7 ± 0.1	7.2 ± 0.1	245 ± 2	318 ± 2
2.5	3.3 ± 0.1	5.6 ± 0.1	273 ± 6	326 ± 7
5	2.3 ± 0.1	4.6 ± 0.1	279 ± 1	343 ± 6

[a]Values are Mean ± SD, n=3. Abbreviations: TF, tissue factor; WB-TG, whole blood thrombin generation; ETPp, endogenous thrombin potential until the thrombin peak.

Supplementary Table S3 The inter-operator variation of the WB-TG assay

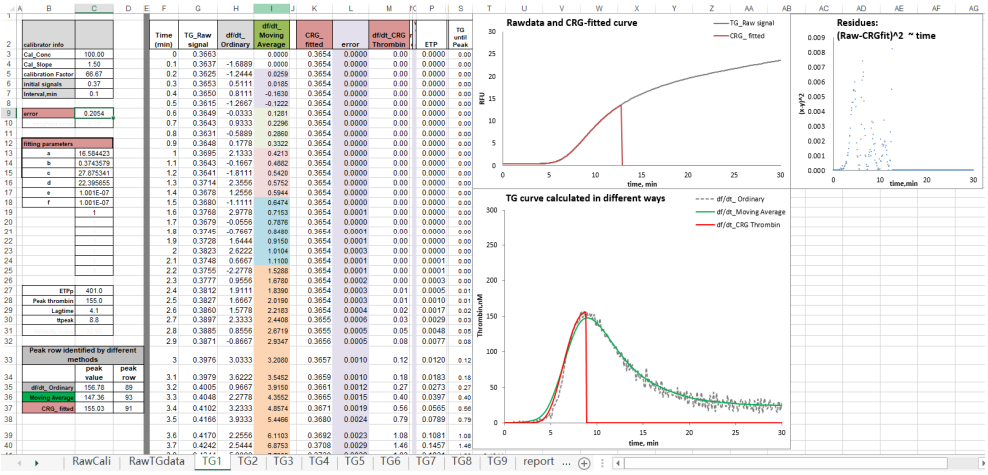
	Operator #1	Operator #2	Operator #3	Operator #4	mean	SD	%CV
Lagtime (min)	4.67	4.87	4.73	3.90	4.54	0.44	9.60
Time-to-peak (min)	11.93	12.03	11.13	9.00	11.03	1.41	12.78
Peak (nmol/L)	74.40	88.85	77.97	94.97	84.05	9.53	11.33
ETPp (nmol*min/L)	333.69	378.54	303.41	288.75	326.10	39.66	12.16

Footnote: The inter-operator variation of the WB-TG assay was determined by letting 4 operators each independently testing a same WB sample. WB-TG was triggered by 1 pmol/L tissue factor, 16.7 mmol/L $CaCl_2$, and 300 µmol/L P2Rho; each condition in triplicates.

Supplementary Table S4 Multivariable linear regression analysis of the determinants of WB-TG parameters

	Lagtime (min)		Peak (nmol/L)		ETPp (nmol*min/L)	
	Beta	P	Beta	P	Beta	P
Erythrocyte count	0.188	0.034	0.205	0.015		
Platelet count					0.159	0.065
Age	-0.307	0.001	0.323	0.001	0.235	0.004
Sex × OC use	-0.229	0.013	0.398	<0.001	0.363	< 0.001

[a]*Sex was defined as male = 0, female = 1 in the analysis. OC use was defined as without OC = 0, with OC = 1. Dependent variables were ETPp, Thrombin-peak, Lagtime. Independent variables were platelet count, erythrocyte count, white blood cell count, mean platelet volume, age, sex, and OC_use. To correct for effect modification, new variables sex ×OC_use, age × OC_use, sex × platelet_count, sex × Erythrocyte_count were also generated and included as independent variable. The "Backward" method was used for the multiple linear regression analysis. Standardized regression coefficients (Beta) of variables in the final model and their P values are shown. Abbreviations: WB-TG, whole blood thrombin generation; ETPp, endogenous thrombin potential until the thrombin peak; OC, oral contraceptive.*



Supplementary Fig.S1 A screen shot of the calculation template for WB-TG. Detailed algorithm is described above.

Intra-assay variation data calculated by 3 operator

calculation by operator#1

	TG1	TG2	TG3	TG4	TG5	TG6	TG7	TG8	TG9	TG10	TG11	TG12	TG13	TG14	TG15	average	SD	%CV
ETPp (nM*min)	241.3	241.4	233.9	245.1	246.5	256.6	236.7	244.1	249.3	246.0	249.2	239.1	225.8	225.5	233.9	**241.0**	**8.7**	**3.6**
Peak (nM)	149.8	152.8	159.5	153.2	154.8	189.0	160.3	158.9	158.5	157.8	163.4	166.1	155.8	160.9	158.9	**160.0**	**9.1**	**5.7**
Lagtime (min)	5.7	5.6	5.6	5.8	5.7	5.6	5.8	5.8	5.6	5.8	5.8	5.6	5.8	5.8	5.7	**5.7**	**0.1**	**1.6**
Ttpeak (min)	8.6	8.5	8.2	8.6	8.6	8.1	8.5	8.6	8.4	8.6	8.5	8.2	8.5	8.4	8.3	**8.4**	**0.2**	**2.0**

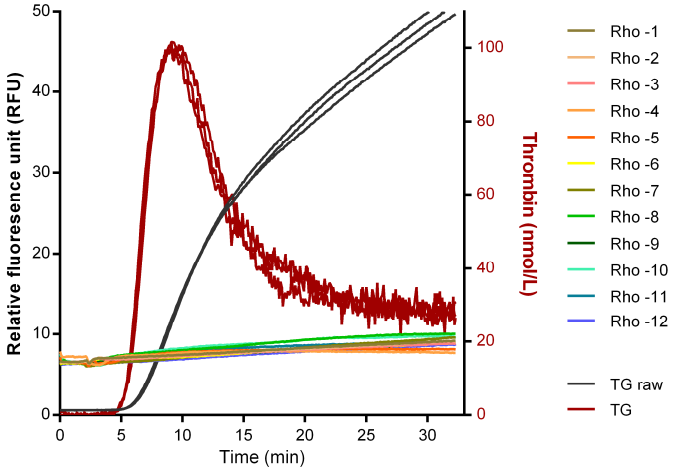
calculation by operator#2

	TG1	TG2	TG3	TG4	TG5	TG6	TG7	TG8	TG9	TG1	TG2	TG3	TG4	TG5	TG6	average	SD	%CV
ETPp (nM*min)	241.3	241.4	233.9	245.1	246.5	256.6	236.7	244.1	249.3	246.0	249.2	239.1	225.8	225.5	233.9	**241.0**	**8.7**	**3.6**
Peak (nM)	149.8	152.8	159.5	153.2	154.8	189.0	160.3	158.9	158.5	157.8	163.4	166.1	155.8	160.9	158.9	**160.0**	**9.1**	**5.7**
Lagtime (min)	5.7	5.6	5.6	5.8	5.7	5.6	5.8	5.8	5.6	5.8	5.8	5.6	5.8	5.8	5.7	**5.7**	**0.1**	**1.6**
Ttpeak (min)	8.6	8.5	8.2	8.6	8.6	8.1	8.5	8.6	8.4	8.6	8.5	8.2	8.5	8.4	8.3	**8.4**	**0.2**	**2.0**

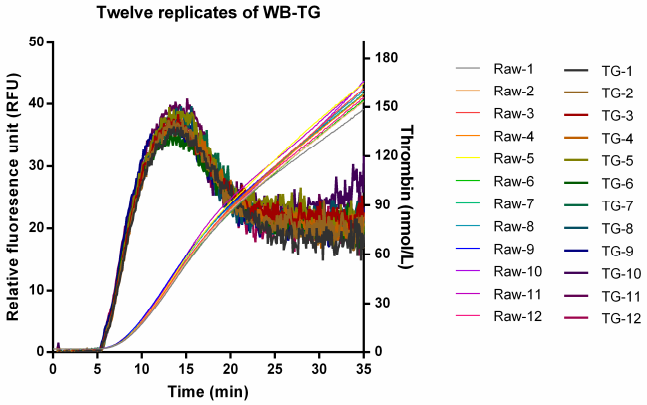
calculation by operator#3

	TG1	TG2	TG3	TG4	TG5	TG6	TG7	TG8	TG9	TG1	TG2	TG3	TG4	TG5	TG6	average	SD	%CV
ETPp (nM*min)	241.3	241.4	233.9	245.1	246.5	256.6	236.7	244.1	249.3	246.0	249.2	239.1	225.8	225.5	233.9	**241.0**	**8.7**	**3.6**
Peak (nM)	149.8	152.8	159.5	153.2	154.8	189.0	160.3	158.9	158.5	157.8	163.4	166.1	155.8	160.9	158.9	**160.0**	**9.1**	**5.7**
Lagtime (min)	5.7	5.6	5.6	5.8	5.7	5.6	5.8	5.8	5.6	5.8	5.8	5.6	5.8	5.8	5.7	**5.7**	**0.1**	**1.6**
Ttpeak (min)	8.6	8.5	8.2	8.6	8.6	8.1	8.5	8.6	8.4	8.6	8.5	8.2	8.5	8.4	8.3	**8.4**	**0.2**	**2.0**

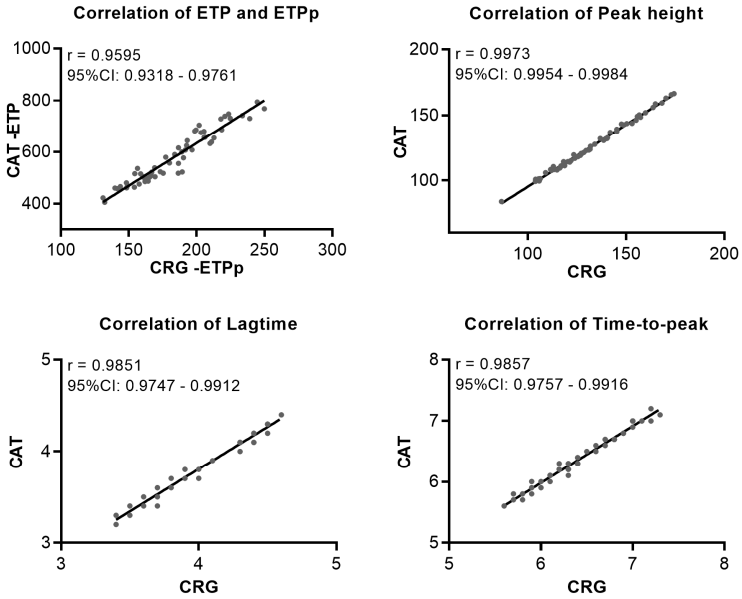
Supplementary Fig.S2 TG parameters calculated by 3 independent operators from 1 set of fluorescence data using the pre-programed calculation template.



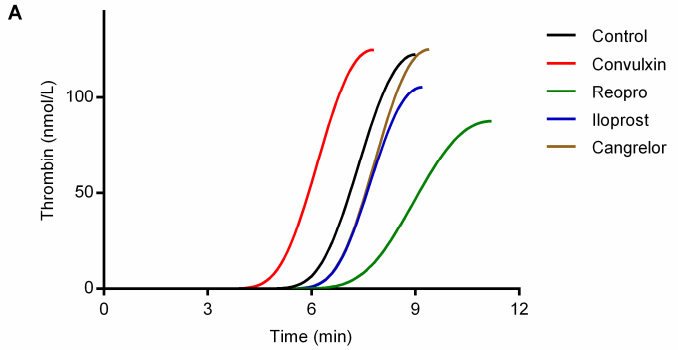
Supplementary Fig. S3 The fluorescence tracing of Rhodamine 110 fluorophore in clotting whole blood. To assess the stability of the quenching effect of clotting WB on fluorescence signal, 2.5 µM Rhodamine 110 was added into clotting WB (triggered by 2.5 pmol/L TF and 16.7 mmol/L $CaCl_2$), and the fluorescence signal was monitored over 30 min. The fluorescence tracing of 12 replicate experiments are shown (lines with rainbow colors), together with the tracing of parallel TG experiments (black lines; triggered by 2.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho) and the corresponding TG curves (dark red lines). The signal of the added Rhodamine110 fluorophore remained relatively stable in the first 0-10 minutes (during which TG reaches the peak), although a slight increase in signal as well as higher variation were found in the later part (20-30 minutes) of the experiments.



Supplementary Fig. S4 The fluorescence tracing of 12 replicate WB-TG experiments. The complete raw fluorescence curves of 12 replicate TG experiments (thin lines; triggered by 2.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho), as well as corresponded TG curves (thick lines), are shown. It can be seen that the TG curves before the thrombin Peak overlapped well between replicates, as well as that of the raw fluorescence data. However, high variation in fluorescence tracing and TG curve are seen in the latter part of the TG experiment.



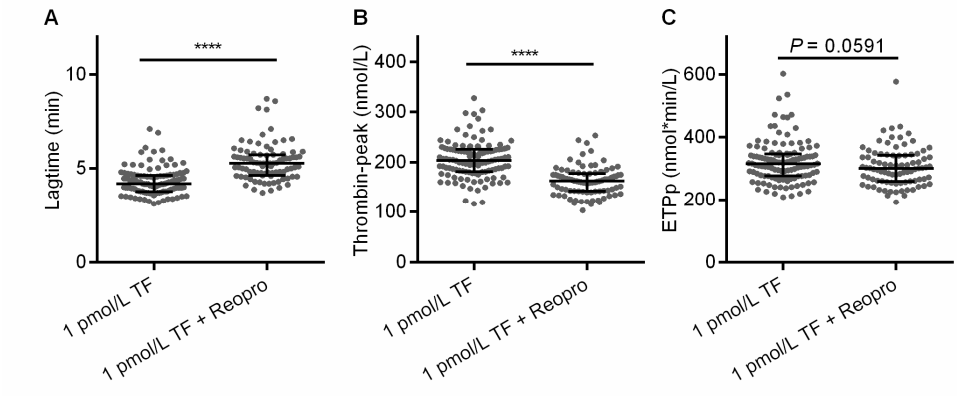
Supplementary Fig. S5 Correlation of thrombogram parameters calculated based on CRG- or CAT-mode. Whole blood samples from 10 healthy donors were tested using the filter paper-based WB-TG method (6 replicate measurements for each sample). From the same data, thrombogram parameters were calculated using both CRG and CAT modes. Spearman correlation coefficients and the 95% confidence intervals (CI) between the TG parameters from the two calculation methods are shown.



B Effect of platelet agonist and inhibitors on WB-TG (n = 3-5)

Parameters	Control	Convulxin	Reopro	Iloprost	Cangrelor
ETPp	100	103.5 ± 3.5	100.7 ± 15.5	96.1 ± 16.8	92.9 ± 7.7
Thrombin-peak	100	102.8 ± 4.3	82.9 ± 11.7	90.0 ± 7.2	98.3 ± 5.1
Lagtime	100	80.1 ± 3.7	115.8 ± 8.5	110.8 ± 0.9	105.7 ± 4.9
Time-to-peak	100	87.5 ± 2.3	116.8 ± 8.2	108.5 ± 6.6	100.7 ± 5.3

Supplementary Fig. S6 Effects of platelet agonist and inhibitors on WB-TG. Blood samples were pre-incubated with Convulxin (50 µg/mL), Reopro (30 µg/mL), Iloprost (10 µmol/L) or Cangrelor (36 µmol/L) for 10 min before TG were triggered with 1 pmol/L TF. (A), representative traces are shown for experiments done in 3 to 5 blood samples. (B), a summary of the effects on WB-TG parameters are shown as percentage of control. Mean ± SD are shown.



Supplementary Fig. S7 Effect of Reopro on WB-TG tested in whole blood samples from 88 healthy donors. WB samples were triggered with 1 pmol/L TF in the presence or absence of 30 µg/mL Reopro. WB-thrombogram parameters Lagtime (A), Thrombin-peak (B) and ETPp (C) are shown. Median and interquartile range are indicated as bars. Comparisons between groups were done using the non-parametric Wilcoxon matched-pairs test; ****: $P < 0.0001$.

CHAPTER 3

Whole blood thrombin generation profiles of patients with cirrhosis explored with a near patient assay

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ABSTRACT

Background & Aims

Patients with cirrhosis have a rebalanced hemostasis, often with normal or elevated thrombin-generating (TG) capacity in plasma. Whole blood (WB) TG allows faster determination and, importantly, includes the influence of all circulating blood cells. We aimed to study the TG profile of patients with cirrhosis in WB and in platelet poor plasma.

Methods

TG in WB and plasma were assessed with a near-patient WB-TG assay and the Calibrated Automated Thrombinography, respectively. TG assays were tested in presence and absence of thrombomodulin. Conventional coagulation tests were also performed.

Results

Thirty-four patients with cirrhosis and twenty-two controls were analyzed. Compared with controls, patients had substantially deranged results in conventional coagulation tests. Comparable WB-TG capacity (endogenous thrombin potential until peak, ETPp) but significantly lower peak thrombin were found in patients, and these results persisted when thrombomodulin was present. TG of the patients was more resistant to thrombomodulin than controls in both WB and plasma, although the inhibitory effect of thrombomodulin was drastically weaker in WB than in plasma. The peak of WB-TG in patients correlated moderately with their hematocrit and platelet count. Significant correlations were found between TG results in WB and plasma.

Conclusions

The WB-TG assay shows a normal to hypocoagulable state in patients with cirrhosis with a decreased anticoagulant activity of TM compared to plasma-TG. The clinical value of this assay needs further validation.

Keywords:

Chronic liver disease; coagulation; thrombin generation; bleeding; thrombosis

INTRODUCTION

Patients with liver dysfunction may acquire substantial alterations in their hemostatic system because the liver is responsible for the synthesis of the majority of hemostatic proteins. Conventional coagulation tests, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis, suggesting a generalized tendency towards bleeding. However, PT and APTT are only sensitive to changes in the procoagulant pathways; therefore reflecting only the abnormalities of the pro-coagulant proteins in cirrhosis, but providing little information about the anticoagulant pathways including antithrombin and proteins of the protein C pathway. Anticoagulant pathways may also be impaired in cirrhosis [1-3]. Clinical data showed that cirrhotic patients are not "auto-anticoagulated", but rather have equal or even higher thrombotic risk than patients without liver disease [2, 4, 5]. These observations have led to the concept of re-balanced hemostasis to explain the complex coagulation profiles in patients with cirrhosis [6-8]. Although a hemostatic balance is reached in cirrhosis, this equilibrium is more fragile and may easily tip towards a hypo- or hypercoagulable state, however traditional coagulation tests are not suitable to assess the functioning coagulation profiles in these patients.

Thrombin generation (TG) is a sophisticated laboratory test capable of assessing the complex interplay between pro- and anti-coagulant pathways, thus reflecting the function of the global hemostatic system. Modern TG assays, such as the Calibrated Automated Thrombinography (CAT) assay, enable continuous TG measurement in plasma and have been shown to be predictive of thrombotic or bleeding risk in various coagulation disorders [9, 10]. The onset, rate and total capacity of TG in a given sample are represented by different parameters of a TG curve, i.e. the lagtime, peak thrombin and endogenous thrombin potential (ETP), respectively [9]. By adding an activator of the anticoagulant protein C pathway, e.g. thrombomodulin (TM) or Protac, TG assay reflects the balance between all pro- and anticoagulant drivers. Studies using the TM-modified TG assays have revealed that the impaired procoagulant system is compensated for by the reduced anti-coagulant function in liver disease. This is reported to occur in both chronic liver disease and acute liver injury/failure [6, 7, 11, 12]. These observations are in agreement with the clinical observations that there is no increased hemostasis-related bleeding risk for most patients.

Although the TM-modified TG assay was a crucial step forward in understanding the net effects of the multiple alterations in coagulation in cirrhosis, there are still several questions that remain to be answered. Standard TG assays are performed with platelet poor plasma (PPP) in the absence of blood cells, with synthetic phospholipids (PL) added to provide surfaces for the assembly of tenase and prothrombinase. These conditions may be different from physiological coagulation, which depends on the surfaces provided by activated platelets and most probably also erythrocytes [13-16]. During physiological coagulation there is a continuous interplay between coagulation initiation, platelet activation, propagation of coagulation and erythrocyte suppletion [13]. In cirrhosis, anemia and thrombocytopenia are common and this could lead to reduced availability of procoagulant surfaces, which could potentially limit the rate and even capacity of TG. A study assessing TG in the platelet rich plasma (PRP) of cirrhotic patients demonstrated

that platelet count is an important determinant of the ETP [17]. Although PRP-TG reflects the influence of platelets, it does not measure the additional impact of other blood cells. On the contrary, whole blood (WB)-TG includes the influence of all circulating blood cells on TG [18]. Another practical advantage of performing WB-TG over plasma TG is that no centrifugation is needed, avoiding potential pre-analytical variation and allowing faster results, which is crucial for point-of-care applications in critically ill or actively bleeding patients [18].

We have previously developed a near patient WB-TG assay by using a miniaturized TG device and a microfluidic chip [19]. This assay was shown to be predictive of post-operative blood loss in patients undergoing elective cardiac surgery [19]. The short turnaround time and compact size of the near patient WB-TG assay system allows possible deployment outside a specialized laboratory environment. In this study, for the first time, we studied WB-TG profiles of a cohort of patients with cirrhosis by using the near patient assay.

MATERIAL AND METHODS

Patients and healthy controls

Patients with cirrhosis and healthy controls were recruited at King's College Hospital NHS Foundation Trust, London. The study was approved by the London (Camden & Islington) Research and Ethics Committee (REC number: 11/LO/0706) and the local Research and Development department at King's College Hospital NHS Foundation Trust. All participants provided written informed consent prior to study entry; where this was not possible, consultee permissions were obtained from their next of kin. Subjects were excluded from the analysis if they had acute liver failure or had received any anticoagulant or anti-platelet therapy.

Blood collection and plasma preparation

Blood was collected into vacuum tubes (1 volume 0.109 M trisodium citrate to 9 volumes blood) (VACUETTE®, Greiner Bio-One, Kremsmünster, Austria). The blood was kept at room temperature and used within 4 h after collection. PPP was prepared by double centrifugation of citrated WB at 2840 g for 10 min. Plasma was aliquoted and frozen at −80 °C before analysis.

WB-TG as measured with a modified near patient assay

WB-TG was measured on a previously described miniaturized TG device[19] and a newly modified microfluidic chip. The previous chip, which was made from methyl methacrylate-acrylonitrile-butadienestyrene (MABS), suffered from occasional fluid leakage.[19] We adopted a new design for the chip, which consisted of two pieces of polydimethylsiloxane (PDMS) with a porous matrix disc of 5 mm in diameter and 190 μm in thickness (grade 589/1; Whatman, Maidstone, United Kingdom) in between. The two PDMS parts were prepared in house by mixing 10 parts of polymer with 1 part of curing agent (Sylgard 184, Dow Corning, Midland, United States) and then solidified in an aluminum mold at 100 °C for 90 min. After treatment in a plasma cleaner (PDC-32G-2, Harrick Plasma, Ithaca, United States) for 15 min, the two PDMS

parts were then assembled with filter paper in the center of the chamber between the two PDMS parts. Permanent bonds formed between the two PDMS parts, hence leakage was effectively prevented.

Citrated blood (15 µL) was mixed with 15 µL trigger solution containing 600 µmol/L fluorogenic thrombin substrate $(ZGGR)_2$-Rhodamine 110 (P_2Rho; Mercachem B.V., Nijmegen, The Netherlands), 5 pmol/L recombinant human tissue factor (TF; Siemens Healthcare, Duisburg, Germany), and 33.4 mmol/L $CaCl_2$ in buffer containing 20 mmol/L HEPES, 140 mmol/L NaCl, and 5 g/L bovine serum albumin (Sigma, Machelen, Belgium) with a pH of 7.35. Fifteen microliter of the above mixture was immediately injected into the inlet of the microfluidic chip and the fluorescence measurement was started. An additional measurement was also done with soluble TM (U-protein Express, Utrecht, The Netherlands) added into the TF-containing trigger solution. The final concentrations were 50% volume blood, 2.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 300 µmol/L P_2Rho in the presence or absence of 150 nmol/L TM. This TM concentration was chosen based on dose-response experiments (Supplementary Fig.1). For every blood sample an independent calibration experiment was performed in which 15 µL α_2-macroglobulin–thrombin complex (α_2M-T, i.e. the calibrator, prepared as previously described[9]) was added to 30 µL of blood and substrate mixture; then 15 µL of the above mixture was injected into the chip and measured. The fluorescence signal was recorded with γ_{ex} =470 nm and γ_{em} = 520 nm. The assay was performed at 37 °C.

From the fluorescence data the WB-TG parameters were calculated using a Chapman-Richard Growth (CRG)-based calculation method as previously described.[19] TG parameters selected for the analyses include the lagtime (the time from triggering coagulation until thrombin is detectable; min), time-to-peak (TTP, the time from initiation until the highest transient thrombin concentration; min), peak (the highest transient thrombin concentration; nmol/L) and endogenous thrombin potential until the thrombin peak (ETPp, i.e. the area under the TG curve until the thrombin peak is reached; nmol×min/L). The TG parameters tested in the presence of TM are depicted as lagtime $^{TM+}$, TTP $^{TM+}$, peak $^{TM+}$ and ETPp $^{TM+}$, respectively. The inter-assay coefficient of variation (CV) of the assay was determined by testing 6 independent measurements per donor, the average inter-assay variation in 5 donors was 7.2%, 2.6%, 14.4% and 13.7% for the lagtime, TTP, peak, and ETPp, respectively.

PPP-TG measurement with the CAT assay

TG in PPP was measured with the CAT technique on a Fluoroskan Microplate Fluorometer (Thermolabsystems OY, Helsinki Finland) as originally described.[9] The final concentration of TF was 5 pM with 4 µM PL in the absence or presence of 10 nmol/L TM. The concentration of TM was chosen to inhibit TG by 50% in normal pooled plasma (NPP). TG parameters were calculated with Thrombinoscope software version 5.0 (Maastricht, The Netherlands), and TG parameters including the lagtime, TTP, peak and ETP were chosen for further analyses.

In each run of TG measurement, NPP was also measured on the same plate. The ETP and Peak values of the study subjects were normalized as the percentage of the ETP and Peak of the NPP tested without TM in the same run, respectively. For better standardization and comparison, the

PPP-TG parameters of samples measured in the presence of TM, including peak $^{TM+}$ and ETP $^{TM+}$, were also normalized as the percentage of the ETP and peak of the NPP tested without TM in the same run. The preparation of the NPP has been described previously.[20] Blood from 116 healthy adult volunteers who gave written consent and did not take any anticoagulant or antiplatelet drugs for at least 2 weeks before blood draw was collected at Maastricht University Medical Center. After an initial centrifugation step (2500g, 5 min) plasma was pooled, followed by ultracentrifugation (100 000g, 10 min). Aliquots of 500 µL were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Standard laboratory measurements

All coagulation assays were performed on the automated analyzer STA-R as previously described.[12] Reagents were obtained from Diagnostica Stago for all coagulation assays which were performed on the automated analyzer STA-R Evolution (Diagnostica Stago). APTT and International Normalized Ratio (INR) were measured by coagulation-based assays, with STA-Cephascreen and STA-Neoplastine, respectively. Clauss methodology was utilized to quantify fibrinogen with STA-Fibrinogen. Antithrombin activity was measured by a chromogenic substrate method, with STA-Stachrom ATIII. Factor (F) II, VIII and X were quantified with STA-deficient II, VIII, X immune-depleted plasmas using coagulation-based assays. Full blood cell count analysis was performed by a flow cytometry method and hemoglobin by a colorimetric method, with reagents from Bayer Diagnostics (Tarrytown, NY, United States) and the Advia 2120 automated hematology analyzer (Bayer Diagnostics).

Statistics

Statistical analyses were performed with SPSS version 25 (Chicago, IL, United States) and graphs were generated using GraphPad Prism software version 6 (La Jolla, CA, United States). Normality of the data was assessed using the Shapiro-Wilk test. Data are represented as median with interquartile range (IQR). Comparisons between two independent groups were performed with the Mann-Whitney test. Fisher's exact test was used to compare nominal data between groups. Spearman test was used for the correlation analysis. A two-sided *P*-value < 0.05 was considered statistically significant.

RESULTS

Characteristics of patients and controls

In total 34 patients with cirrhosis and 22 healthy donors were included in the analyses. As shown in Table 1, the patient group was older ($P < 0.001$) but had similar gender distribution compared with controls. The etiology of cirrhosis included alcohol related liver disease (n=17), non-alcoholic fatty liver disease (n=7), viral hepatitis (n=3) and primary sclerosing cholangitis (n=3). In addition, drug induced liver injury, primary biliary cirrhosis, non-alcoholic steatohepatitis and autoimmune disease were each found in one patient.

Four patients were classified as Child-Pugh class A, 18 patients were in class B, and 12 patients in class C. The median Model for End-Stage Liver Disease (MELD) score of the patients was 17.5 (IQR: 14.3-23.8). Twenty-six patients had acutely decompensated (AD) cirrhosis, with a CLIF-AD score of 51 (45-53). Six patients fulfilled criteria for chronic liver failure (ACLF), of which 3 patients had a CLIF-ACLF grade 2, 3 patients had grade 3 [21, 22].

Conventional coagulation tests and cell counts

Patients with cirrhosis had significantly distorted coagulation profiles compared to healthy controls, as shown in Table 1. Patients had significantly higher INR and longer APTT ($P < 0.001$ for both). Lower levels of both pro- and anticoagulant factors were found in the patients, such as FII, FX and antithrombin except FVIII which was higher ($P < 0.001$ for all factors compared with control). Clauss fibrinogen was comparable between the patients and controls.

Platelet count and hematocrit of the patient group were noticeably lower than the local reference ranges. The median (IQR) values in the patients were 92.5 (59-159) $\times 10^9$/L and 0.32 (0.27-0.37) for platelet count and hematocrit, respectively, whereas the respective local reference ranges were 150-450 $\times 10^9$/L and 0.36-0.50. The white blood cell counts of the patients were within the local reference range. The cell counts of the controls were not measured.

Table 1 Characteristics and coagulation profiles of patients with cirrhosis and healthy controls

	Patients		Controls		*P* value
	n	Median (IQR)	n	Median (IQR)	
Age, years	34	58 (53.3 - 63)	22	32.5 (28 - 39.5)	<0.001
Gender: female	34	10(29.4%)	22	12 (54.5%)	0.093
Etiology					
ALD		17 (50%)			
NAFLD		7 (20.6%)			
Viral hepatitis		3 (8.8%)			
PSC		3 (8.8%)			
other		4 (11.8%)			
Laboratory tests					
Sodium, mmol/L	34	136 (134 - 138)			
Creatinine, μmol/L	34	79 (68 - 109)			
Albumin, g/L	34	31.5 (26 - 34)			
Bilirubin, μmol/L	34	43.5 (23.3 - 109)			
Child-Pugh classification					
A		4 (11.8%)			
B		18 (52.9%)			
C		12 (35.3%)			
MELD score	34	17.5 (14.3 - 23.8)			
Coagulation parameters					
Platelet, $x10^9$/L	34	92.5 (59 - 159)			
Hematocrit	34	0.32 (0.27 - 0.37)			
Hemoglobin, g/L	34	99.5 (88 - 118)			
White cell count, $x10^9$/L	34	4.65 (3.43 - 6.53)			
INR, ratio	25	1.47 (1.26 - 1.81)	20	1.02 (0.98 - 1.07)	<0.001
APTT, second	22	38.8 (35.2 - 43.3)	19	29.4 (28.6 - 31.5)	<0.001
Fibrinogen, g/L	25	2.8 (1.72 - 4.15)	20	2.8 (2.5 - 3.12)	0.802
FII, %	25	53 (42 - 72)	20	98 (88.3 - 105)	<0.001
FVIII, %	26	242 (210 - 284)	20	118 (93 - 175)	<0.001
FX,%	25	55 (47 - 67)	20	93.5 (87.8 - 108)	<0.001
AT, %	27	57 (38 - 81.5)	20	104.5 (99 - 107.5)	<0.001

Footnote: values are median (interquartile range) or number (percentage). Abbreviations: IQR, interquartile range; ALD, alcoholic liver disease; NAFLD, nonalcoholic fatty liver disease; PSC, primary sclerosing cholangitis; MELD, the Model for End-Stage Liver Disease; INR, international normalised ratio; APTT, activated partial thromboplastin time; FII, factor II; FVIII, factor VIII; FX, factor X.

WB-TG profiles

Fig. 1A shows representative WB-thrombograms of the patients and controls. As summarized in Table 2 and shown in Fig. 1B, patients had significantly longer lagtime and TTP than controls ($P < 0.01$ for both; Fig. 1B). The peak thrombin value was substantially lower in patients than in controls (medians values were 118 and 179 nmol/L for patients and controls, respectively) ($P < 0.001$). However, the ETPp was not significantly different between the patients and controls (230 [196-294] versus 247 [222–280] nmol×min/L, respectively) ($P = 0.356$).

The addition of 150 nmol/L TM into the WB-TG assay substantially prolonged the lagtime in both patients and controls to a similar extent of 37% ($P = 0.926$). Conversely, the added TM only exhibited a weak inhibitory effect on ETPp. TM-induced inhibition on the ETPp was slightly lower in patients at 10.3 (2.0-19.9) % than in controls at 18.9 (12.7-27.5) % ($P = 0.048$). In the presence of TM, the ETPp $^{TM+}$ was still similar between patients and controls ($P = 0.709$), whereas the peak $^{TM+}$ remained lower in patients ($P < 0.001$) (Table 2 and Fig.1C).

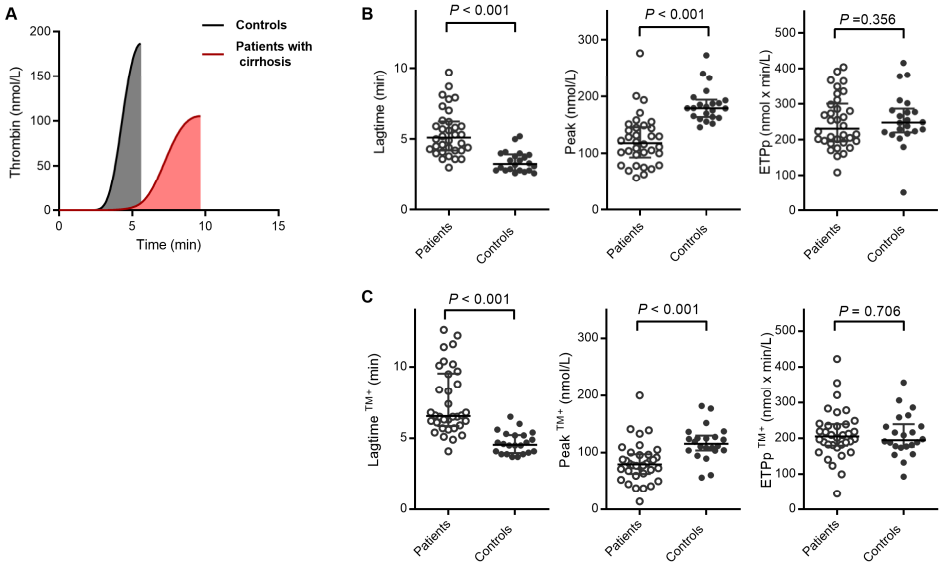

Fig.1 Whole blood thrombin generation profiles of the study subjects. The whole blood thrombin generation profiles of patients with cirrhosis and healthy controls were studied with a near patient assay at 2.5 pmol/L tissue factor. (A), representative curves of WB-TG of the patients (n=34) and controls (n=22) are shown. WB-TG parameters in the absence and presence of thrombomodulin (TM) are shown in (B) and (C), respectively. Mann-Whitney test was used to compare between groups, *P* values are shown. Bars in the graph represent the median and interquartile range.

Table 2 Whole blood thrombin generation profiles of patients and controls

	Patients (n=34)	Controls (n=22)	*P* value
WB-TG parameters in the absence of TM			
Lagtime (min)	5.1 (4.2 - 6.1)	3.3 (2.8 - 3.9)	<0.001
TTP (min)	8.4 (7.3 - 9.9)	5.7 (5.1 - 6.8)	<0.001
Peak (nmol/L)	118 (98 - 145)	179 (164 - 192)	<0.001
ETPp (nmol×min/L)	230 (196 - 294)	247 (222 - 280)	0.356
WB-TG parameters in the presence of TM			
Lagtime $^{TM+}$ (min)	6.6 (6.1 - 9.3)	4.6 (4.1 - 5.2)	<0.001
TTP $^{TM+}$ (min)	10.9 (9.8 - 15)	8.1 (7.1 - 8.9)	<0.001
Peak $^{TM+}$ (nmol/L)	79.7 (65.1 - 96.2)	115 (104 - 127)	<0.001
ETPp $^{TM+}$ (nmol×min/L)	205 (181 - 238)	195 (177 - 233)	0.709

Footnote: values are median (interquartile range). Abbreviations: WB, whole blood; TG, thrombin generation; TM, thrombomodulin; TTP, time to peak; ETPp, endogenous thrombin potential until the thrombin peak.

PPP-TG profiles

We obtained TG data from 28 out of the 34 patients, because PPP was not collected from 5 patients, and no TG curve was obtained from one of the patients. When PPP-TG was measured in the absence of TM, comparable lagtime and TTP values were found in patients and controls. The peak and ETP were slightly but significantly lower (12.2% and 14% lower in median values, respectively) in patients (Table 3 and Fig. 2A). When PPP-TG was tested in the presence of TM, the peak $^{TM+}$ and ETP $^{TM+}$ values of the patient plasma were significantly higher than the controls (35.8% and 58.7% higher in median values; $P = 0.009$ and $P < 0.001$, respectively) (Table 3 and Fig. 2B).

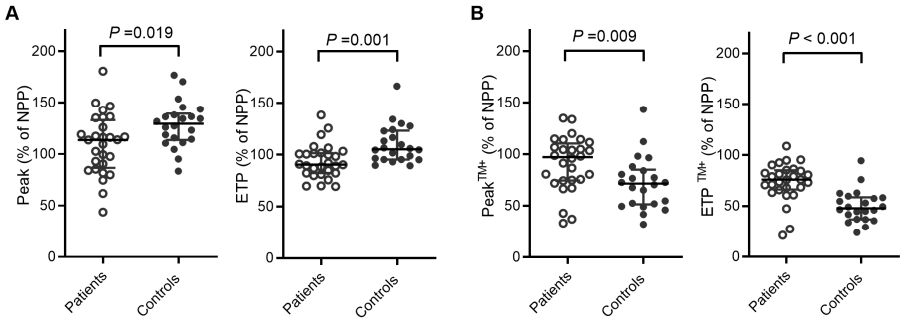

Fig. 2 Plasma thrombin generation profiles of the study subjects. Platelet poor plasma of patients with cirrhosis (open circles) and healthy controls (filled dots) were tested with the calibrated Automated Thrombinography (CAT) assay at 5 pmol/L tissue factor, 4 μmol/L phospholipids in the absence or presence of 10 nmol/L thrombomodulin (TM). The peak thrombin and ETP values in the absence (A) and presence (B) of thrombomodulin (TM) are shown. All peak and ETP values both in the absence and presence of TM were normalized as the percentage of that of the normal pooled plasma (NPP) tested without TM in the sample run. Mann-Whitney test was used to compare between groups.

Table 3 Plasma thrombin generation profiles in the absence and presence of thrombomodulin

	Patients (n=28)	Controls (n=22)	*P* value
PPP-TG parameters in the absence of TM			
Lagtime (min)	2.3 (2 - 3.3)	2.3 (2.3 - 2.6)	0.627
TTP (min)	4.3 (3.8 - 5.3)	4.7 (4.3 - 5.1)	0.082
Peak (% of NPP)	113 (89.1 - 128)	129 (115 - 137)	0.019
ETP (% of NPP)	90.5 (82.3 - 101)	105 (96 - 121)	0.001
PPP-TG parameters in the presence of TM			
Lagtime $^{TM+}$ (min)	2.7 (2.3 - 3.6)	2.4 (2.3 - 2.7)	0.043
TTP $^{TM+}$ (min)	4.5 (4.1 - 5.3)	4.3 (4 - 4.5)	0.102
Peak $^{TM+}$ (% of NPP)	96.9 (74.6 - 108)	71.4 (52.1 - 83.1)	0.009
ETP $^{TM+}$ (% of NPP)	75.8 (67.4 - 84.5)	47.8 (38.1 - 58)	<0.001

Footnote: values are median (interquartile range); the peak and ETP values both in the absence and presence of TM were all normalized as the percentage of that of the NPP tested without TM in the sample run. Abbreviations: PPP, platelet poor plasma; TG, thrombin generation; TM, thrombomodulin; TTP, time to peak; NPP, normal pooled plasma; ETP, endogenous thrombin potential.

Correlations of TG parameters with other factors

We explored a possible relation between TG parameters and other factors. Age of the study subjects were counterintuitive correlated with longer lagtime (r = 0.435) and TTP (r = 0.405), as well as lower peak (r = -0.301) of WB-TG, but not with ETPp (r = 0.021, *P* = 0.879) (Table 4). The PPP-TG parameters in the presence of TM also showed moderate to weak correlations with age, including the lagtime $^{TM+}$ (r = 0.394) and ETP $^{TM+}$ (r = 0.284).

Moderate to weak correlations were found between WB-TG parameters in the absence of TM with corresponding PPP-TG parameters in absence of TM, including the lagtime (r = 0.282), peak (r = 0.468) and ETP (r = 0.415) (Table 4).

Positive correlations were found between the hematocrit of patients and their WB-TG parameters, including the peak (Spearman r = 0.434), ETPp (r = 0.417) (Table 4) and peak^{TM+} (r = 0.374). The peak value was also positively correlated with the platelet count of the patients (r = 0.559). The platelet count showed weaker correlation with the ETPp (r = 0.304, *P* = 0.085).

In addition, the platelet count was positively correlated with some parameters of the PPP-TG, including the lagtime (r = 0.529, *P* = 0.004), ETP (r = 0.45, *P* = 0.016) and lagtime $^{TM+}$ (r = 0.528, *P* = 0.004). No significant correlation was found between hematocrit and PPP-TG parameters.

Cirrhosis severity, as represented by the Child-Pugh classification and MELD score, was correlation with longer lagtime and lower peak of WB-TG, both in the absence and presence of TM (*P* < 0.05 for all correlations). The above scores were also correlated with lower peak of PPP-TG (*P* < 0.05 for both correlations).

Table 4 Correlations between WB-TG parameters and other factors

	WB-TG parameters					
	Lagtime	Peak	ETPp	Lagtime^{TM+}	Peak^{TM+}	ETPp^{TM+}
Age	0.435**	-0.301*	0.021	0.408**	-0.207	0.033
Platelet count	-0.147	0.559**	0.304	-0.346*	0.342	0.027
Hematocrit	-0.162	0.434*	0.417*	-0.289	0.374*	0.335
PPP-TG parameters						
Lagtime	0.282*	0.251	0.410**	0.219	0.156	0.079
Peak	-0.344*	0.468**	0.280*	-0.397**	0.460**	0.057
ETP	-0.295*	0.629**	0.415**	-0.432**	0.568**	0.121
Lagtime^{TM+}	0.449**	0.046	0.350*	0.397**	0.000	0.100
Peak^{TM+}	-0.008	-0.250	-0.178	0.042	0.006	0.009
ETP^{TM+}	0.241	-0.537**	-0.258	0.295*	-0.237	0.050

*Footnote: Spearman correlation coefficients are shown. * p<0.05; ** P<0.01. Abbreviations: WB, whole blood; TG, thrombin generation; TM, thrombomodulin; PPP, platelet poor plasma; ETPp, endogenous thrombin potential until thrombin peak; ETP, endogenous thrombin potential.*

DISCUSSION

We studied the TG profiles of patients with cirrhosis in WB using a near patient WB-TG assay and in PPP with the standard CAT assay. WB-TG results showed normal TG capacity but lower TG rate in patients with cirrhosis, suggesting normal- to hypocoagulability; whereas TM-modified PPP-TG suggested hypercoagulability, with both higher TG capacity and rate found in cirrhosis. The WB-TG parameters showed moderate correlations with platelet count and hematocrit, as well as the standard PPP-TG parameters. We also surprisingly found that WB-TG is less sensitive to the anticoagulant function of TM than PPP-TG.

TG assays have been extensively used to study the coagulation profiles of patients with liver disease, and studies using TM-modified plasma TG assay have shown intact or elevated TG potential in cirrhosis, supporting the concept that these patients have re-balanced hemostasis [6, 23-27]. However, until now all studies on TG profiles in these patients were performed in plasma samples, which has limitations, such as the requirement for a specialized laboratory environment, a longer turn-around time for results and the inability to study the effect of most blood cells (except platelets) on TG. Until now there are no published data of WB-TG measurements in liver disease patients. Although whole blood viscoelastic assays have been tested in liver disease patients and shown additional value in reducing blood product administration, they do not assess the protein C pathway, and their ability to predict clinical outcome is still unclear [28, 29].

Using the near patient WB-TG assay, we observed that the endogenous thrombin generating potential (ETPp) values of patients with cirrhosis, regardless of the presence/absence of TM, were in the same range as in healthy controls, suggesting that these patients had normal thrombin-generating capacity. The standard PPP-TG results add to the growing literature that hypocoagulable state is suggested when TG is tested without activating the protein C pathway, whereas hypercoagulable state is found when the function of the anticoagulant protein C pathway is included in TG by adding TM into the test [6, 24-27, 30]. These results indicate that cirrhosis does not necessarily result in a hypocoagulable state as suggested by their lower procoagulant factor levels and prolonged PT and APTT, thus reinforcing that prophylactic administration of fresh-frozen plasma according to their PT/APTT is not required [31, 32].

Of note, although the TG capacity of cirrhotic patients is comparable to the controls, their peak thrombin level is discordantly lower, suggesting a hypo-coagulable state. The ETP and Peak are usually well correlated in PPP-TG, but their relation in WB has not been extensively explored. The above discordance might be due to a differential effect of low cell count on peak and ETPp. In our experience and in literature, the platelet count much more affects the peak of PRP-TG than the ETP. In fact the ETP plateaued once the platelet count reached a certain level (approximately $100*10^9$ platelets/L) but the peak still increases with further ascending platelet counts [33]. The influence of erythrocyte count on WB-TG is less explored, but a recent study found that at a normal platelet concentration of $200*10^9$/L the peak of WB-TG was significantly augmented by increasing hematocrit from 0.2 to 0.45, whereas the ETP was largely unchanged [34]. Therefore, the lower counts of platelets and erythrocytes in cirrhotic patients might have induced differential impact on the peak and ETP of WB-TG, and caused the above observed discordance. Platelets and erythrocytes are involved in TG *via* many mechanisms, including but not limited to their expression of procoagulant surface [13, 14, 35-37]. The activation/function of platelets in cirrhosis remains controversial [38]. It has been reported that platelets are defective in aggregate formation, have a storage pool defect and defective transmembrane signaling in cirrhosis [39], while other studies have reported that blood cells from cirrhotic patients have abnormally high levels of PS exposure and this may contribute to their hypercoagulable state [40, 41]. Moreover, erythrocytes, as the most abundant cells in circulation, may also indirectly impact TG by augmenting platelet activation [36, 42]. Overall the function of blood cells in TG of liver disease patients is still poorly characterized and this WB-TG assay has potential to improve insight into this topic.

Interestingly, WB-TG showed less sensitivity to the anticoagulant function of TM than PPP-TG. A high dose (150 nmol/L) of TM only induced weak inhibition on WB-TG, as the ETPp in patients and controls was only inhibited by 10.3% and 18.9%, respectively. Although the WB-TG of cirrhotic patients showed increased resistance to TM compared to controls, the difference was only on the border of statistical significance ($P = 0.048$). Conversely, a much lower dose of TM (10 nmol/L) exhibited a much stronger inhibitory effect on the ETP of PPP-TG and the effect was 5-fold weaker in the patients than in controls. The TM resistance in cirrhosis is most probably due to their reduced protein C and protein S, combined with the elevated FVIII levels [43-45]. The high dose of TM, however, did substantially prolong the lagtime of WB-TG in both

patients and controls, but this effect may be not *via* the anticoagulant function of the protein C pathway, but rather because TM prevented thrombin from activating platelets and consequently limited the availability of procoagulant phospholipids [46].

This study has several limitations. Firstly, the number of study subjects was relatively low and a heterogenous group of patients was included. Secondly, the age of the cirrhotic patients was higher than the healthy controls. However, the effect of age on thrombin generation in the general population is modest, and in patients with cirrhosis this effect is even smaller as cirrhosis-related distortions of the coagulation system outweigh the effects of age on plasma levels of coagulation factors [26]. This is further supported by our observation that older age was counterintuitively correlated with longer lagtime and lower peak of WB-TG. Furthermore, due to unknown reasons, WB-TG was more resistant to TM than PPP-TG despite used at a high concentration. It is noteworthy that Lebreton *et al.* reported that plasmatic hypercoagulability of cirrhosis patients was detectable only when testing TG with the addition of TM but not activated protein C [26]. Further understanding of the function of TM in WB-TG is warranted to further optimize the TM-modified WB-TG assay for better evaluation of the protein C system. Future studies with larger sample size will be needed to explore the feasibility of this assay to assist coagulation management in liver disease patients.

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Author contributions

T.W.Ow, L.Rabinowich & O.Barbouti recruited participants, collected samples and patient data. J.Wan, W. Hendrix & J. Konings performed thrombin generation experiments. R.Arya, W.Bernal, T.Lisman, L.N.Roberts & M.Roest designed and supervised the study. V.C.Patel & B.de Laat assisted with analyses and writing of the paper. J.Wan, W.Bernal, T.Lisman, L.N.Roberts & M.Roest interpreted the data and wrote the manuscript. All authors approved the final version for submission.

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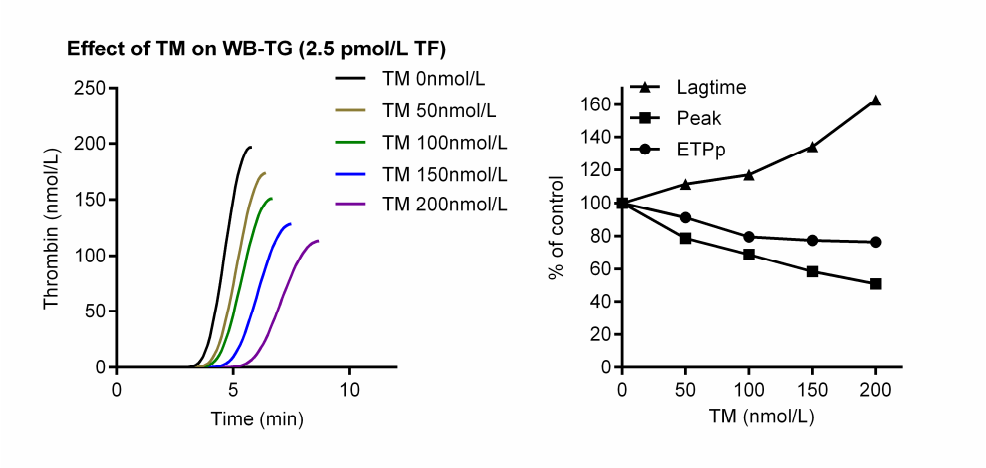
SUPPLEMENTARY DATA



Supplementary Fig.S1. Effects of different concentrations of thrombomodulin (TM) on whole blood thrombin generation (WB-TG). WB-TG triggered by 2.5 pmol/L tissue factor (TF), 16.7 mmol/L $CaCl_2$ and 300μmol/L thrombin substrate P_2Rho in response to different concentrations of TM (from 0 to 200 nmol/L) are shown. The right panel shows the relative changes of lagtime, peak and ETPp to TM.

CHAPTER 4

Erythrocytes impair the anticoagulant function of the protein C system in whole blood thrombin generation

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In preparation

ABSTRACT

Background & Aims

Dysfunction of the protein C system, an important regulatory pathway, increases the risk of thrombosis. Previous studies have shown that the activity of activated protein C (APC) depends on the type of cell surfaces. This study aims to explore the influence of erythrocytes on the anticoagulant function of APC and thrombomodulin in thrombin generation (TG).

Methods

The impact of thrombomodulin on WB-TG was tested in 119 healthy donors. The effects of APC and thrombomodulin on calibrated TG were tested in WB, platelet rich plasma (PRP), and in platelet poor plasma (PPP) reconstituted with variable amounts of erythrocytes. The effects of synthetic phospholipids supplementation on the activity of APC in PPP- and WB-TG were assessed.

Results

Erythrocyte counts of 119 healthy donors, but not their platelet or leukocyte counts, were inversely correlated with the inhibitory effect of thrombomodulin on WB- endogenous thrombin potential until peak (ETPp) ($r = -0.41$, $p<0.001$). Supplementation of APC or thrombomodulin dose-dependently prolonged the lag time and reduced the ETPp in both PRP- and WB-TG. Interestingly, the anticoagulant effects of APC and thrombomodulin on ETPp and peak were both approximately 3 times less effective in WB than in PRP. Supplementation of washed erythrocytes into PPP dose-dependently suppressed the inhibitory effect of APC on ETPp, but the addition of synthetic phospholipids into WB dose dependently enhanced the inhibitory effect of APC.

Conclusions

Erythrocytes impaired the anticoagulant effect of APC, but not the generation of APC in WB. This may partly explain the increased thrombotic risk related with high erythrocyte count or erythrocyte transfusion.

Keywords:

Anticoagulant; protein C; thrombomodulin; whole blood; thrombin generation

INTRODUCTION

Coagulation is a tightly regulated process, involving complex interactions between pro- and anticoagulant enzymes and their co-factors. The protein C system is an important anticoagulant driver, consisting of the plasmatic factors protein C and protein S, as well as endothelial receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR)[1]. During coagulation, TM-bound thrombin activates protein C to activated protein C (APC); APC, together with its cofactor protein S, cleaves and inactivates activated factor V (FVa) and FVIIIa, two important cofactors in the prothrombinase and tenase complexes, respectively, to prevent excessive thrombin generation (TG). Elevated thrombotic risk has been related with protein C/S deficiency or a poor response to APC, also known as APC resistance, often seen in FV Leiden carriers or women taking oral contraceptives.[1, 2]

Blood cells are important regulators of the protein C system. Endothelial cells provide receptors such as TM and EPCR that facilitates APC generation by up to 20000-fold and 20-fold, respectively.[3, 4] Furthermore, APC cleaves FVa more efficiently on human microvascular endothelial cells than on platelets.[5] Platelet activation induces APC resistance as shown by the increased ratio of activated prothrombin time (APTT) with versus without APC[6], in line with the finding that platelets sustain procoagulant events by providing a membrane surface that delays cofactor inactivation and by releasing a cofactor molecule that displays an APC-resistant phenotype.[7] In addition, platelet factor 4 (PF4), a protein that exist abundantly in platelet α-granule, inhibits FVa cleavage by APC in purified systems and impairs the inhibition of endogenous thrombin potential (ETP) by APC in platelet poor plasma (PPP)-TG.[8] However, the overall effect of PF4 on the anticoagulant function of the protein C system in physiological coagulation remains unclear because PF4 was also shown to interact with TM and the γ-carboxyglutamic acid domain of protein C[9] and accelerates the activation of protein C by the thrombin-TM complex both in vitro[10] and in vivo[11].

Erythrocytes, the most abundant cells in circulation, have long been considered as a bystander in coagulation but recent studies implied that they are directly involved in coagulation. Clinically, increased erythrocyte count (polycythemia) is associated with increased risk of both arterial and venous thrombosis[12-14]. The prothrombotic effect of high erythrocyte count was mainly attributed to its influence on blood rheology[13] but it was also shown that approximately 0.5% of erythrocytes in circulation express negatively charged phospholipids (PL) that can support prothrombinase activity[15]. On the other hand, erythrocyte membrane and erythrocyte-derived vesicles support the inactivation of FVa by APC[16, 17]. In addition, erythrocytes also promote platelet activation, which may affect TG, as well as the function of the protein C pathway in blood[13]. For example, intercellular adhesion molecule-4 (ICAM4)[18] and Fas receptor (FasR)[19] on the erythrocyte membrane interacts with platelet integrin αIIbβIII and Fas ligand (FasL), respectively, and promote platelet p-selectin expression and phosphatidylserine (PS) exposure. Due to these complex mechanisms, the overall effect of erythrocyte on the function of the protein C pathway is still largely unknown.

We recently developed a fluorogenic whole blood (WB)-TG assay to measure the function of plasma coagulation factors and the involvement of platelets and erythrocytes in tissue factor (TF)-induced TG[20]. A modification of this assay with supplemented soluble APC or TM gives insight about the anticoagulant activity of the protein C system on WB-TG. Interestingly, we observed that the anticoagulant function of TM was drastically weaker in WB-TG than in PPP-TG, to an extend that may not be solely explainable by platelet-related factors alone[21]. In the current study, we explored the overall influence of erythrocytes on the anticoagulant effect of the protein C system in WB-TG.

MATERIAL AND METHODS

Study subjects and blood sample preparation

Blood was collected into vacutainer tubes (with 3.2% sodium citrate; BD Vacutainer System; United Kingdom) from healthy adults who did not take any anticoagulant two weeks prior to the blood collection, after giving full informed consent according to the Helsinki declaration. This study was approved by the Medical Ethical Committee of Maastricht University Medical Center, as previously described [20].

Blood was kept at room temperature and used within 4 hours after collection. Platelet rich plasma (PRP) was prepared by centrifugation of blood at 220g for 15 minutes, and the platelet counts were determined on a Coulter Counter analyzer (Beckman Coulter; United States). PPP was prepared by double centrifugation of citrated blood at 2840g for 10 minutes.

Washed erythrocytes were prepared by washing the residual pellet after PRP preparation three times in HEPES buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% w/v glucose, and 0.1% w/v bovine serum albumin, pH 7.4) and centrifuged at 330g for 10 minutes after the first two washing steps and at 890g for 10 minutes after the third washing step. Only minimal residual platelets and leukocytes were found when measured on a Coulter Counter analyzer.

The anticoagulant effect of TM on WB-TG tested in 119 healthy donors

WB-TG of 119 healthy donors was measured as described previously [20] with 1 pM tissue factor (TF; Siemens Healthineers AG, Germany), 16.7 mM $CaCl_2$, 300 μM $(ZGGR)_2$-Rhodamine 110 (Stago, Gennevilliers, France) with and without the addition of 150 nM TM (Synapse Research Institute, Maastricht, the Netherlands). The fluorescence signal was monitored continuously with 6 seconds interval on a Fluoroskan Ascent microplate fluorometer (Thermolabsystems, Finland) with λex = 485 nm and λem = 538 nm using Fluoroskan Ascent Software (version 2.6). All steps were carried out at 37ºC. TG parameters, including the lagtime, time-to-peak, thrombin peak and endogenous thrombin potential until thrombin peak (ETPp) were calculated from the fluorescence data as originally described [20].

The anticoagulant effect of TM or APC on TG in WB, PRP and reconstituted blood

To compare the anticoagulant effects of TM/APC in WB- and PRP-TG, measurements were performed according to our recently published protocol[20] with some minor modifications. The originally described fluorogenic thrombin substrate $(ZGGR)_2$-Rhodamine 110 was replaced with ZGGR-AMC (Bachem, Switzerland) because the latter is more widely available and still gave acceptable signal. In short, citrated WB/PRP was activated by mixing with 1 pM TF, 16.7 mM $CaCl_2$, 416.7 μM ZGGR-AMC, with and without the addition of various concentrations of TM (10, 20, 40, 80 or 160 nM) or APC (4, 8, 16, 32 or 64 nM; prepared as described[22]). The measurement setting was identical as above mentioned except that the fluorescence recording was with λex = 390 nm and λem = 460 nm. A H-transform[23] was used to correct the data when substrate consumption and inner filter effect was obvious, i.e. in plasma.

To study the influence of erythrocyte counts on the anticoagulant effect of TM/APC on TG, washed erythrocytes were supplemented into autologous PPP to make the final erythrocyte count 0, 1.75, 3.5 or $5*10^{12}$/L. TG was then trigged by 1 pM TF, 16.7 mM $CaCl_2$, 417 μM ZGGR-AMC and 1 μM synthetic phospholipids (PL) with 20 mol% phosphatidylserine (PS), 20 mol% phosphatidylethanolamine (PE) and 60 mol% phosphatidylcholine (PC) (Avanti Polar Lipids Inc., United States), in the presence or absence of 60nM TM or 8 nM APC.

To study whether the anticoagulant effect of TM/APC on WB-TG was influenced by synthetic PL, in some WB-TG experiments PLs was supplemented into WB at serial concentrations (0, 1, 2 or 4 μM) before TG was triggered with 1 pM TF, 16.7 mM $CaCl_2$, 417 μM ZGGR-AMC, in the presence or absence of 150 nM TM or 16 nM APC.

PPP-TG measurement with the classical CAT assay

PPP-TG was measured with the Calibrated Automated Thrombography (CAT) as originally described by Hemker et al[22]. In short, TG in PPP was triggered by 1 pM TF, 16.7 mM $CaCl_2$, 417 μM ZGGR-AMC and varying concentrations of phospholipids (0, 0.25, 0.5, 1, 2, 4 or 8 μM), in the presence or absence of 10 nM TM or 5 nM APC. Fluorescence signal was measured on a Fluoroskan Ascent microplate fluorometer with λex = 390 nm and λem = 460 nm at 37 ºC. TG parameters, including endogenous thrombin potential (ETP) and peak, were acquired using Thrombinoscope software version 5.0 (Thrombinoscope BV, the Netherlands).

Statistics

Statistical analyses were performed using GraphPad 5.0 (Graphpad Software, San Diego, United States). Results are expressed as mean±SD or median and interquartile range as indicated. The correlation between the anticoagulant activity of TM and the count of erythrocytes in the healthy population was tested with the Spearman test.

RESULTS

Correlation between erythrocyte count and the anticoagulant effect of TM on WB-TG

We tested WB-TG in the absence or presence of TM in 119 healthy donors. The TM concentration was chosen based on a dose dependent curve and the chosen concentration induced 25.4 ± 6.3% inhibition on ETPp and prolong the lagtime by 84.1 ± 15.6%. Interestingly, we observed a moderately inverse correlation (spearman r = -0.41, p < 0.001; Fig.1) between the inhibition rate of ETPp by TM and the erythrocyte count of the donors; whereas no significant correlation was found between the inhibition of ETPp by TM and the counts of platelets or leucocytes (p=0.109 and 0.232, respectively), indicating that erythrocytes could diminish the anticoagulant effect of TM in WB-TG.

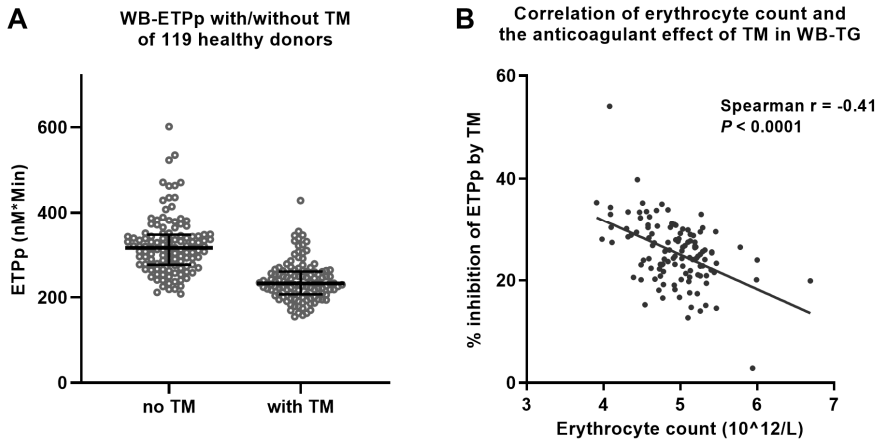

Fig.1 The anticoagulant effect of thrombomodulin in relation to the erythrocyte counts of 119 healthy donors. (A) The ETPp of WB-TG tested in the absence and presence of thrombomodulin (TM) of 119 healthy individuals. TG was triggered by 1 pM tissue factor, 16.7 mM $CaCl_2$ in the presence of 300 μM thrombin substrate P_2Rho and with or without 150 nM TM. Bars in the graph represent median and interquartile range. (B) The correlation between erythrocyte counts of the donors and the inhibition rate of ETPp by TM, as determined by the Spearman test.

The anticoagulant effects of APC and TM were both less effective in WB- than in PRP-TG

We compared the impact of APC/TM supplementation on TG in WB and PRP. Ascending APC concentrations induced increased anticoagulant effect in a dose-dependent manner in both PRP- and WB-based TG experiments, as shown by the prolonged lagtime, reduced peak and ETPp (Fig.2 A&B). However, the anticoagulant effect of APC on TG was 2 to 3 times weaker in WB than in PRP. In fact, 64 nM APC induced 32.5 ± 2.3% reduction of peak and 24.4 ± 7.5% decrease of ETPp when added into WB-TG, while causing, respectively, 73.6 ± 1.2% and 71.7

± 0.4% reductions on the peak and ETPp of PRP-TG. Supplementation of TM into PRP- and WB-TG resulted in similar results, with higher anticoagulant effect of TM seen in PRP compared to in WB (Fig.2 C&D). Interestingly, the TM-induced ETPp inhibitions were also 2 to 3 times weaker in WB than in PRP, indicating that erythrocyte only influenced the APC activity but not APC generation by thrombin-TM complex.

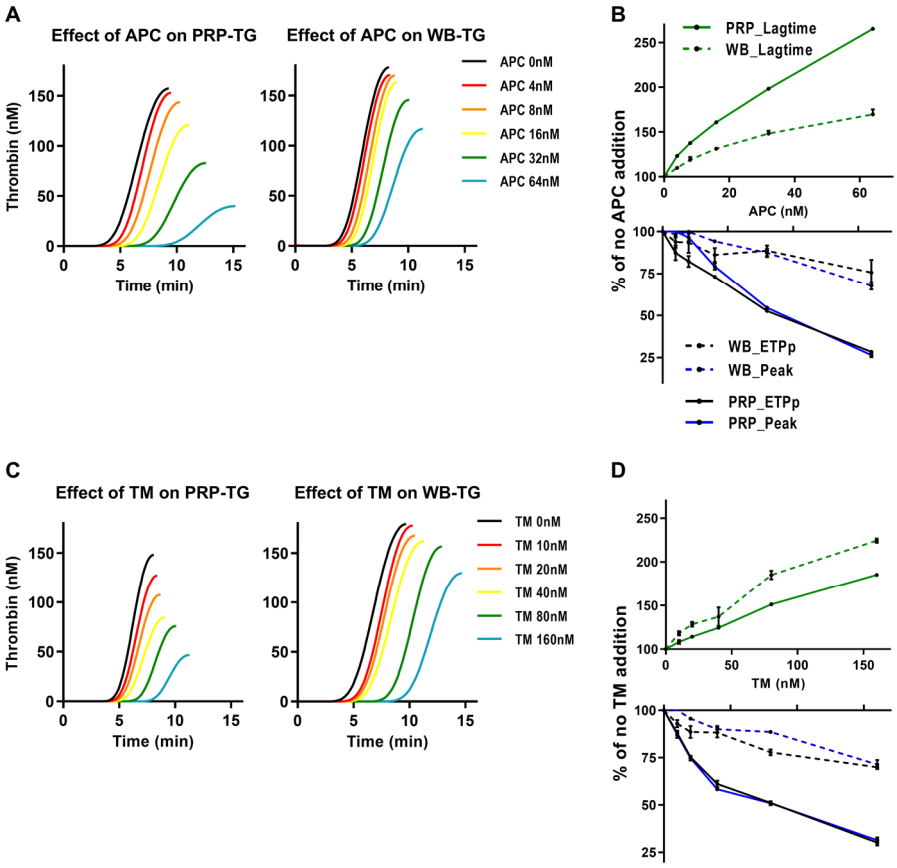

Fig.2 Comparison of the anticoagulant effect of the protein C pathway (activated protein C [APC] and thrombomodulin [TM]) in platelet rich plasma (PRP) and whole blood (WB) thrombin generation (TG). The PRP and WB were from the same donor. (A) and (C) presents representative curves of TG that was triggered by 1 pM tissue factor, 16.7 mM $CaCl_2$, 417μM ZGGR-AMC in the absence or presence of APC (4, 8, 16, 32 or 64 nM) or TM (10, 20, 40, 80 or 160 nM), respectively. The relative changes of PRP- and WB-TG parameters in response to APC or TM are shown in (B) and (D), respectively.

Erythrocyte count dose dependently impairs the anticoagulant effect of APC and TM on TG

Based on the above results, we developed the hypothesis that erythrocytes are the major cause of the different anticoagulant effect of TM/APC in PRP- and WB-TG. We next tested this hypothesis by comparing the anticoagulant effect of TM and APC in plasma reconstituted with different counts of erythrocytes. Although the addition of washed erythrocyte into PPP increased peak thrombin of TG (as previously observed[20]), it also induced dose-dependent impairment of the inhibitory effect of APC on ETPp from 86.7 ± 1.7% (0 erythrocyte/L) to 31.2 ± 5.5% ($5*10^{12}$ erythrocyte/L) (Fig.3). The effect of TM on ETPp was similarly impacted by the addition of erythrocytes (Fig.3).

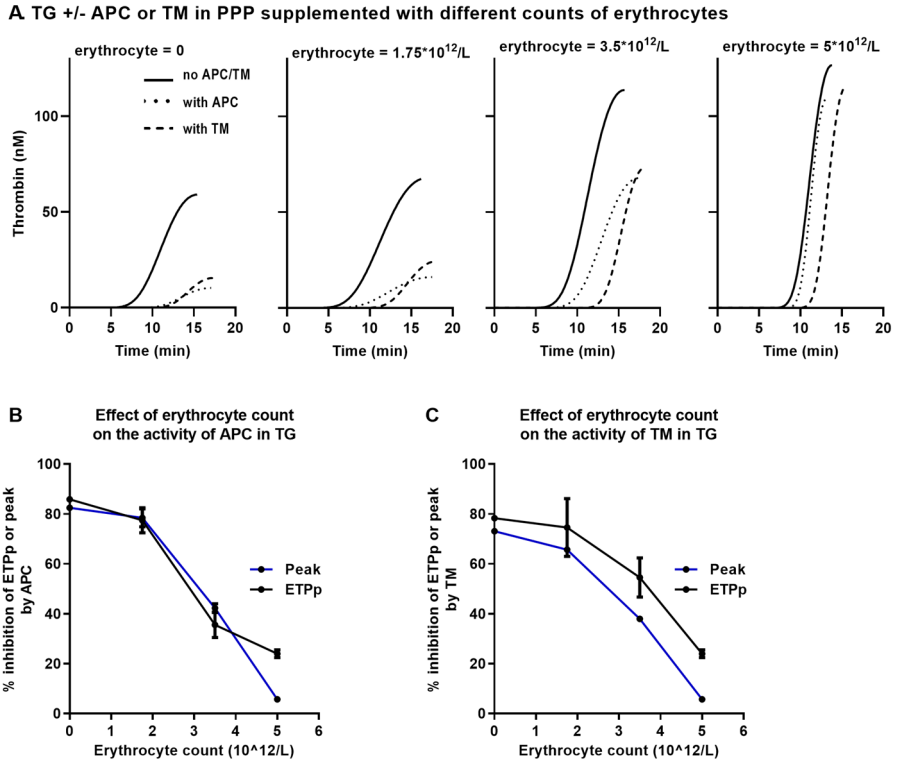

Fig.3 Influence of erythrocyte counts on the anticoagulant effect of APC or TM in TG. Plasma was reconstituted with washed erythrocytes to reach different erythrocyte counts (0, 1.75, 3.5 or 5*10^12/L). (A) presents representative curves of TG that was triggered by 1pM tissue factor, 16.7mM $CaCl_2$, 1μM synthetic phospholipids, 417μM ZGGR-AMC and in the absence or presence of 8nM APC or 60nM TM. The % inhibition of ETPp and peak thrombin by APC or TM are shown in (B) and (C), respectively.

Supplementation of synthetic phospholipids increases the anticoagulant effect of TM/APC in TG

Similar to previous studies,[24, 25] we observed that the anticoagulant efficacy of APC in PPP-TG was dependent on the concentration of synthetic PL used in the reactions (Supplemental Fig.1). The inhibition rate of ETP by APC increased from 38% in absence of PL supplementation to 100% when PL was added at 4μM.

We also explored whether the supplementation of synthetic PL could influence the function of the protein C system in WB-TG reaction. In line with the findings in PPP-TG, the inhibition of WB-ETPp by APC was drastically enhanced from 24.2% with no synthetic PL supplementation to 63.6% when 4 μM PL was added into the reaction (Fig.4). A similar trend was observed with the inhibitory effect of TM in WB-TG when synthetic PL was added (Fig.4).

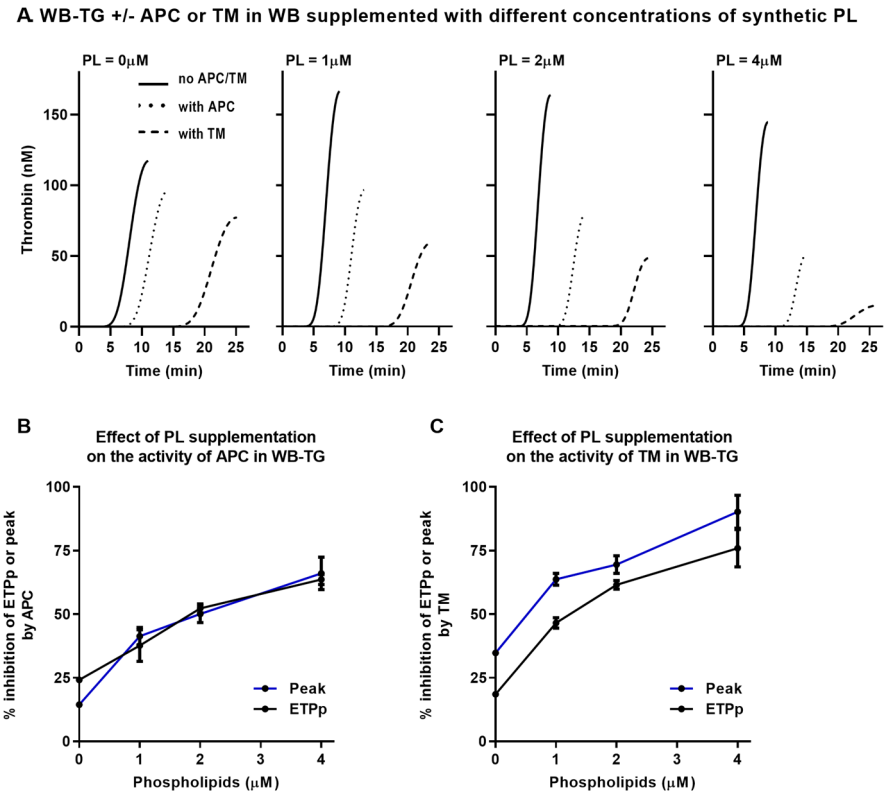

Fig.4 The effect of different doses of phospholipid (PL) supplementation (0, 1, 2, or 4 μM) on the anticoagulant effect of APC/TM in WB-TG. (A) WB-TG was triggered by 1pM tissue factor, 16.7 mM $CaCl_2$, 417 μM ZGGR-AMC in the absence or presence of 16 nM APC or 150 nM TM. The inhibition rates of ETPp or peak by APC or TM are shown in (B) and (C) respectively.

DISCUSSION

The findings that platelets impact both the procoagulant pathways (via receptor binding, granule content release and PS exposure)[26] and the anticoagulant protein C pathway (through the release of PF4[8], protein S[27] and a special pool of FVa[7]) implies a multifaceted role of blood cells in regulating coagulation. The current study shows that the anticoagulant effect of APC and TM in WB-TG is impaired by erythrocytes, suggesting that erythrocytes also play important roles in regulating the protein C system.

Our findings of a negative correlation between the inhibitory effect of TM on WB ETPp and erythrocyte counts, but not platelet or leukocyte counts, in a population of 119 healthy individuals indicated that erythrocytes may regulate coagulation in WB via inhibition of the protein C pathway. Further in vitro experiments showed that the anticoagulant effects of APC and TM were 2 to 3 times weaker in WB- than in PRP-TG, which supported the influence of erythrocytes on the protein C pathway. Given that the activity of APC and TM were reduced to a similar extend in WB compared with in PRP, these cells most likely exert their influence by impairing the activity of APC but not through reducing APC generation by thrombin-TM complex. Our hypothesis that erythrocytes impair the anticoagulant function of APC was confirmed by the finding that the anticoagulant effect of APC and TM decreased gradually when PPP was supplemented with increasing numbers of erythrocytes.

Protein C resistance is an important risk factor for thrombosis, as seen in FV Leiden and oral contraceptive use[2]. Based on our finding here that erythrocyte cause APC resistance, it is very likely that the WB of individuals with polycythemia is more resistant to APC compared with people with normal erythrocyte counts. Therefore, our finding might have filled a knowledge gap on the mechanism of the high thrombotic risk in these patients[14] because classical coagulation tests, mostly tested in plasma, may only give an incomplete picture on the regulation of coagulation. Similar theory may also be applied to chronic obstructive pulmonary disease[35] and clinical procedures like erythrocyte transfusion [36].

The adverse effect of erythrocytes on APC's anticoagulant effect is not platelet dependent. Erythrocytes can induce platelet activation [13, 18, 19], which, as afore mentioned, may indirectly impair APC activity. However, this effect may not be the determining role here because we showed that erythrocytes added into PPP, in the absence of platelets, also diminished the anticoagulant effect of APC on TG. Another possible mechanism may be related to erythrocytes' ability of augmenting TG. As shown in our previous study[20] and the current study, an increase of erythrocyte count in reconstituted blood leads to increased thrombin peak. Based on our experience that stronger TG (e.g., when TG is triggered with higher concentration of tissue factor) requires higher concentration of APC/TM to reach a similar extend of TG inhibition, it is possible that erythrocytes increased the difficulty for APC to restrain TG. However, this hypothesis is in contradiction to our observation that synthetic PL supplementation increased the anticoagulant effect of APC in both PPP- and WB-TG despite that it also augmented thrombin peak.

The PL composition of erythrocyte outer leaflet membrane may contribute to the suppressing effect of erythrocytes on APC activity. Previous studies showed that the activity of APC can be enhanced by certain PL, in particular PE,[29] which exist abundantly (20% mol) in synthetic PL. On the contrary, a recent study that combined phospholipase digestion with mass spectrometric lipidomics found that PE only account for 5.8% of the PL composition in the outer leaflet of erythrocytes with the majority of PE exist in the inner leaflet [31]. This difference in PL composition might have render erythrocyte membrane more suitable for supporting procoagulant reactions while being a inadequate surface for APC.

Compared with PPP-TG which relies on synthetic PL to provide procoagulant surface, WB-TG is a better representer of physiological coagulation because it depends on physiological PL from blood cells. A limitation of our study is that that the influence of endothelial cell function is not fully represented in this test. We are aware that although the addition of TM/APC in in vitro experiments resembles part of the endothelial function, the physiological anticoagulant function of the protein C pathway is activated by thrombin-thrombomodulin complex in a receptor mediated process on endothelial cells [5, 30]. However, APC may also be incorporated into a clot by diffusion or in cases of APC infusion; thus, our WB-TG assay could serve as a model for the function of APC in a red venous clot.

In conclusion, the current study shows that the anticoagulant activity of APC is impaired by an increasing number of erythrocytes, which may partly explain the increased thrombotic risk related with high erythrocyte counts (polycythemia or chronic obstructive pulmonary disease) or erythrocyte transfusion.

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Author contributions

J. Wan, J. Konings, M. Roest conceptualized the study; J. Wan performed the experiments and analyzed results with J. Konings; J. Wan drafted the manuscript with comments from all coauthors; all authors checked the final version and agree with the submission.

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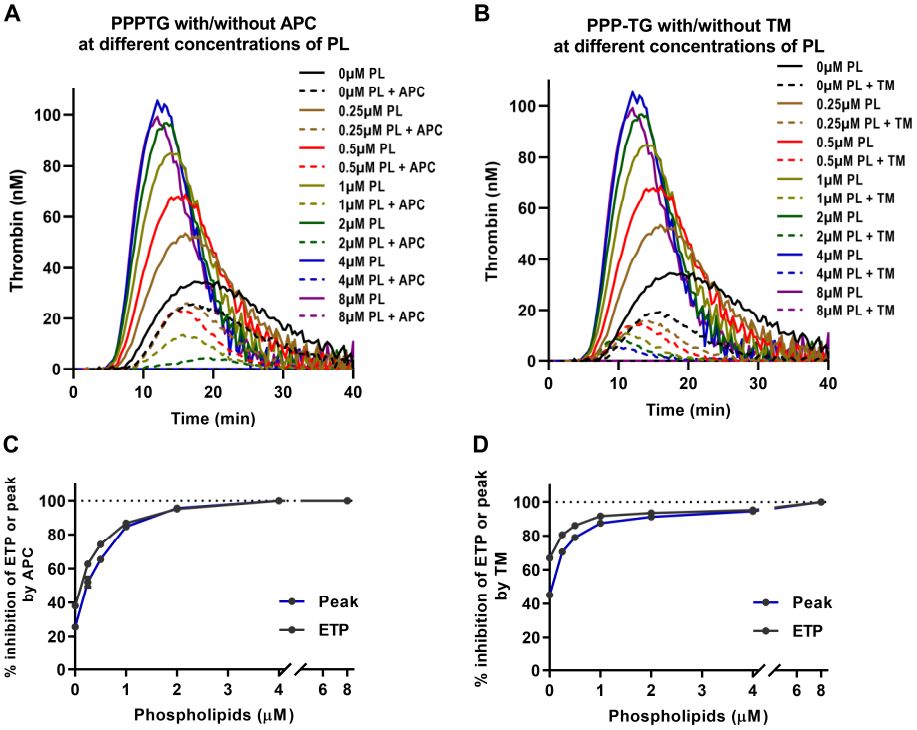
SUPPLEMENTAL INFORMATION



Supplemental Fig.S1 Effect of phospholipid (PL) concentrations on the anticoagulant effect of APC or TM on PPP-TG. TG was triggered with 1pM tissue factor, 16.7mM CaCl2, 417μM ZGGR-AMC and different doses of phospholipids (60%PC/20%PS/20%PE; 0 to 8 μM as indicated in the figures), and in the absence or presence of 5nM APC (A) or 10nM TM (B). The inhibitory effect of APC/TM on TG in response to different concentrations of PL are shown in (C) and (D), respectively.

CHAPTER 5

Added value of blood cells in thrombin generation testing

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Thromb Haemost; in press

ABSTRACT

The capacity of blood to form thrombin is a critical determinant of coagulability. Plasma thrombin generation (TG), a test that probes the capacity of plasma to form thrombin, has improved our knowledge of the coagulation system and shows promising utility in coagulation management. Although plasma TG gives comprehensive insights in the function of pro- and anticoagulation drivers, it does not measure the role of blood cells in TG. In this literature review, we discuss currently available continuous TG tests that can reflect the involvement of blood cells in coagulation, in particular the fluorogenic assays that allow continuous measurement in platelet rich plasma and whole blood. We also provide an overview about the influence of blood cells on blood coagulation, with emphasis on the direct influence of blood cells on TG. Platelets accelerate the initiation and velocity of TG by phosphatidylserine exposure, granule content release and surface receptor interaction with coagulation proteins. Erythrocytes are also major providers of phosphatidylserine and erythrocyte membranes trigger contact activation. Furthermore, leukocytes and cancer cells may be important players in cell-mediated coagulation because, under certain conditions, they express tissue factor, release procoagulant components and can induce platelet activation. We argue that testing TG in the presence of blood cells may be useful to distinguish blood cells-related coagulation disorders. However, it should also be noted that these blood cells-dependent TG assays are not clinically validated. Further standardization and validation studies are needed to explore their clinical usefulness.

Key words: thrombosis; hemorrhage; thrombin generation; blood cells; whole blood.

INTRODUCTION

The blood coagulation system consists of a serial of coagulation factors and cofactors that are separated from their physiological activators by the endothelium under normal conditions [1-4]. Upon exposure to subendothelial TF [5] or intravascular TF [6-10], coagulation could be triggered by the formation of the factor(F) VII-TF complex, which then triggers the formation of a tiny amount of thrombin through the activation of FIX and FX on a negatively charged surface [11-14]. Thrombin amplifies its own formation *via* positive feedback loops involving the activation of FXI, FVIII and FV [15, 16]. The subsequent burst of thrombin converts fibrinogen into fibrin monomers, which polymerize and are cross-linked into a dense clot to seal the wound. Meanwhile, thrombin also limits its own production through negative feedback by acting together with the endothelial receptor thrombomodulin to activate the anticoagulant protein C. Activated protein C (APC), together with cofactor protein S, cleaves FVIIIa and FVa to regulate the amount of thrombin generated [13, 14, 17, 18]. Blood cells, most notably platelets, also contribute to the formation of a blood clot. Once in contact with subendothelial collagen, platelets get activated, form aggregates and expose procoagulant surfaces to support thrombin generation (TG) [19-21].

Assessment of blood coagulability is essential for the clinical management of replacement therapy or anticoagulation in the settings of hemophilia and thrombophilia, respectively. Traditionally, this was done primarily by monitoring fibrin clot formation after TG is initiated with high concentrations of extrinsic or intrinsic activators through prothrombin time (PT) or activated partial thromboplastin time (APTT) measurements [22, 23]. A limitation of testing fibrin formation is that it occurs at very low levels (~5%) of thrombin formation [24, 25], while important physiological information after the TG initiation phase is not represented. Indeed, PT and APTT are insensitive to changes in the anticoagulant pathways, and are therefore not indicative for thrombotic risks caused by anticoagulant pathway impairment [25].

In contrast to PT and APTT, TG assays (TGAs) are usually initiated with low concentrations of TF, and report the full process of thrombin activation and inactivation. Furthermore, TG can be further optimized for specific pathway testing such as the protein C pathway by addition of thrombomodulin [26, 27]. Nowadays, TG is mainly tested in platelet poor plasma (PPP-TG), with exogenous synthetic phospholipid vesicles added to mimic the physiologic procoagulant surfaces. Although PPP-TG gives, in many aspects, deeper insights in coagulation than PT and APTT, it does not represent the involvement of blood cells, such as the platelets, erythrocytes and leukocytes. By testing TG in platelet rich plasma (PRP-TG), many influences of platelets on coagulation have been revealed (*vide infra*). However, the impact of other cells is still unclear, primarily due to the lack of assessment tools. In this review, we discuss currently available TG assays, especially those capable of testing TG in presence of blood cells (i.e. in PRP or whole blood [WB]). In addition, we summarize effects of blood cells on coagulation and highlight the direct influences of these cells on TG testing. Advantages and limitations of these blood cell-dependent TG tests will also be discussed.

PPP-TGA: PRINCIPLE, ADVANTAGES, AND PITFALLS

History and principle of TG assays

TGA was pioneered in 1953 by Macfarlane and Biggs [28], who subsampled an activated blood sample at regular time intervals into test tubes containing fibrinogen, and then calculated the thrombin activity at each time point by comparing the respective clotting time with a calibration curve. The time- and labor-consuming nature of the assay was drastically improved in 1993 by Hemker *et al.* through the introduction of a slow-reacting chromogenic thrombin substrate, which allowed thrombin activity to be continuously measured by monitoring the cleavage of the substrate, without the need for timed subsampling [29]. Although chromogenic methods allow continuous TG measurement, fibrin formation in plasma needs to be prevented to avoid disturbance on optical density detection. This is usually done by defibrinating PPP or by adding an inhibitor of fibrin polymerization into the test; both are known to influence TG [30, 31]. Hemker and colleagues solved this problem by introducing a fluorogenic thrombin substrate which is not disturbed by fibrin formation (Fig. 1) [32, 33]. Issues related to fluorescence monitoring such as the inner filter effect, substrate consumption or the influence of the color of plasma on fluorescence, were solved by introducing a parallel calibration experiment in which substrate cleavage by the α_2-macroglobulin-thrombin complex with a constant thrombin activity in the same plasma is recorded [32, 34]. A dedicated H-transform algorithm was developed to calibrate against the above mentioned issues to allow objective calculation [35]. This fluorogenic TG assay is commercially known as the Calibrated Automated Thrombography (CAT) which allows relatively fast and high throughput measurement of up to 48 samples per run in PPP and PRP, a tremendous improvement compared to the subsampling method of one man-hour per curve [32, 34].

Currently, there are several commercially available semi-automated chromogenic TGAs [36], including HemoScan Thrombin Generation Assay (HemoScan), Pefakit in-TDT (Pentapharm) and Innovance ETP (Siemens Healthcare). Semi-automated fluorogenic TGAs include Technothrombin-TGA (Technoclone) and CAT (Diagnostica Stago). There are also two fully automated fluorogenic TG assays, i.e. the CEVERON-TGA (Technoclone) and ST-Genesia (Diagnostica Stago).

Thrombogram parameters

The whole course of prothrombin conversion and thrombin inactivation over time is shown as a thrombogram, which is typically characterized by 5 parameters (Fig.1B). The time needed for thrombin to reach a detectable concentration is defined as lag time. The highest transient thrombin concentration during the reaction is referred to as peak height, and the time needed to reach this peak is called time to peak. Velocity index (VI) can be obtained by dividing peak height by the difference between time to peak and lag time. Finally, the area under the TG curve, which represents the total amount of thrombin activity during the reaction, is termed endogenous thrombin potential (ETP).

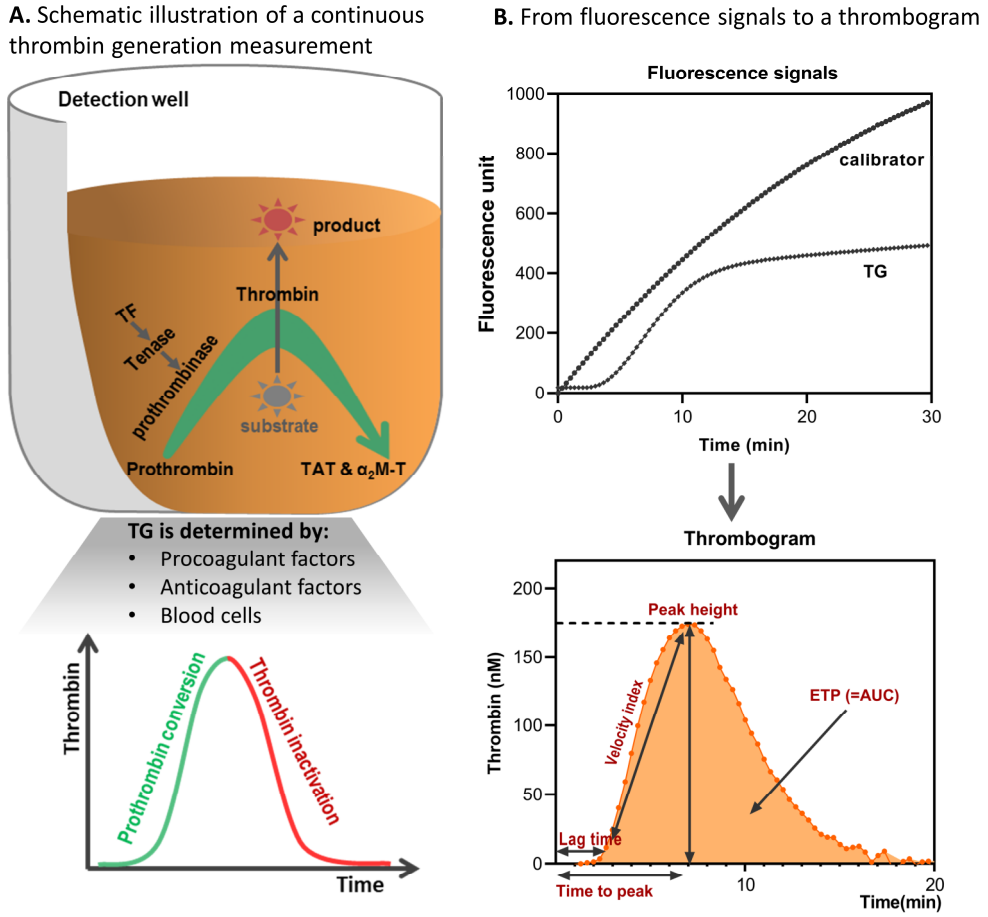

Fig. 1 Overview of the thrombin generation (TG) assay. (A) a schematic illustration of fluorogenic thrombin generation assays. After coagulation is triggered with tissue factor (TF) in a recalcified blood sample, the complex interactions between pro- and anticoagulant factors as well as blood cells leads to prothrombin conversion. The generated thrombin cleaves a fluorogenic thrombin substrate and result in an increase in fluorescence signal. Thrombin is inactivated by its natural inhibitors in blood into antithrombin-thrombin (TAT) and α_2Macroglobulin-thrombin (α_2M-T) complexes. (B) the fluorescence signals of TG experiments are used to generate a thrombogram, which can be characterized by the so-called TG parameters, including lag time, time to peak, thrombin peak, velocity index and endogenous thrombin potential (ETP, i.e. the area under the TG curve [AUC]).

Clinical utilities of PPP-TG

The clinical utility of PPP-TG assay has been extensively explored and reviewed in detail elsewhere [25, 37, 38]. In brief, the PPP-TG parameters have been shown to be predictive of the risk of idiopathic venous thromboembolism (VTE) [39] and VTE recurrence [40], as well as the amount of blood loss after cardiac surgery [41]. TGAs also have the potential to serve as a

laboratory tool to monitor replacement therapy in hemophilia [42] or anticoagulant treatments [43]. In addition, TGAs are useful tools for exploring novel mechanisms of hemostasis or thrombosis. For example, thrombomodulin- or APC- modified TGAs were essential tools for the establishment of prothrombotic effect of oral contraceptive pills [44, 45], as well as the rebalanced pro- and anti-coagulant coagulation system in liver disease [26, 46].

Limitations of PPP-TG assays

In the early development phase of the TG assay, there were major concerns about the high inter-center variation of PPP-TG, which render the assay not suitable for clinical laboratories. Later, it was shown that acceptable imprecision can be obtained given that standardized reagents and good thermal control are guaranteed, and that preanalytical conditions, including blood collection method, the use of a contact pathway inhibitor, transportation method, storage time and centrifugation protocol, are standardized [47-51]. The use of a reference plasma to normalize the TG parameters further improved the inter-laboratory variation [52, 53]. Recently introduced fully automated TG testing systems are good tools for standardized TG and are expected to bring TG to the routine repertoire of tests conducted in hemostasis centers.

Table 1. Characteristics of TG in PPP, PRP and WB.

	PPP-TG	PRP-TG	WB-TG
Advantage	Commercially available semi-automated assays and fully automated assays	Commercially available semi-automated assays	Includes all circulating cells, therefore reflects the influences of these cells on TG
	High through-put	Reflects the influence of platelet dysfunction on TG	Less influenced by pre-analytical variations than plasma tests
	Good standardization	Reflects the influence of some platelet-associated factors (e.g. VWF, FXI)	Fast measurement (potential for point-of-care use)
			Low blood volume required
Disadvantage	Blood cells are omitted in the test, thus not possible to study cell mediated thrombosis or bleeding	Erythrocytes, leucocytes (and cancer cells) are omitted	There is no commercial assay or standardized reagent
	Centrifugation is needed to prepare PPP from WB, therefore has a long turn-round time	Pre-analytical variations during PRP preparation and long turn–around time	Lack of standard sample for normalization and influence of blood flow or endothelium is not included
	Influence of blood flow or endothelium are not included	Lack of standard sample for normalization and influence of blood flow or endothelium is not included	

Abbreviations: TG, thrombin generation; PPP, platelet poor plasma; PRP, platelet rich plasma; WB, whole blood; VWF, von Willebrand factor; FXI, factor XI.

Despite the above-mentioned improvements, PPP-TGA still has some limitations (summarized in Table 1). One major limitation of PPP-TG is that the physiological procoagulant surfaces, i.e. blood cells, are not available in plasma, while instead synthetic phospholipids are used at saturated concentration to resemble the function of blood cells. As a result, the blood cell-dependent variations between individuals are not studied with this method. It is now widely established that physiological hemostasis follows a cell-based model, instead of the classical models which divides hemostasis into primary and secondary hemostasis [14, 54]. The cell-based model describes a three-phase interplay between blood cells and thrombin generation. First, TF-bearing cells initiate TG and lead to the activation of a small amount of thrombin (i.e. the initiation phase). Thrombin then activates platelets to expose phosphatidylserine, as well as activating FXI, FV and FVIII (the amplification phase); these processes prepare necessary procoagulant surface and reactants to allow a burst of thrombin formation during the "propagation phase". The influence of blood cells on TG is not limited to providing phospholipids, but also via many other mechanisms (Fig. 2). TGA that includes the blood cells, either in PRP or WB, may be useful for studying the cellular influences in coagulation disorders and, may allow more comprehensive coagulation profiling.

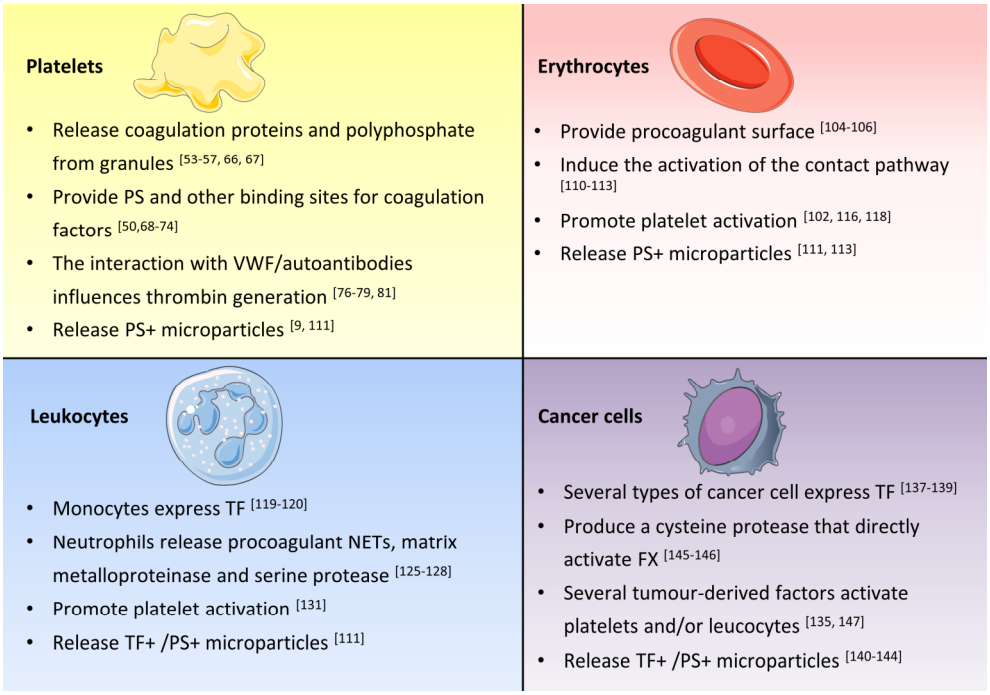

Fig. 2 Brief summary of the influence of blood cells on thrombin generation (TG). Blood cells affects TG through many mechanisms, including the exposure of rocoagulant phosphatidylserine (PS), releasing of granule contents, production of PS- and/or tissue factor (TF)-positive microparticles, or through the localization of coagulation factors to membrane receptors, etc. NETs, neutrophil extracellular traps.

PRP-TGAS AND THE INFLUENCE OF PLATELETS ON TG

PRP-TG was first explored by subsampling methods [28], which, although was time- and labor-consuming, provided early evidences for a role of platelets in TG [55]. The fluorogenic assays largely avoids unwanted platelet activation caused by frequent subsampling and are now the most widely used method in the field.

Influence of platelet granule secretion on PRP-TG

Platelets possess two kinds of storage organelles, the α-granules and dense granules; the content of these granules are released to the surrounding environment upon platelet activation [56, 57]. Polyphosphates, stored in platelet dense granules, may enhance the activation of FV and FXI by thrombin. In addition, it was also reported that polyphosphate nanoparticles released onto the platelet surface cause the (auto)activation of FXII, thus potentially enhancing TG and fibrin clot formation [58, 59]. Coagulation factors secreted from the α-granules, including FV, prothrombin, fibrinogen, Von Willebrand Factor (VWF), high molecular weight kininogen and FXIII, promote TG and fibrin clot formation. Platelets also secret anticoagulant proteins such as tissue factor pathway inhibitor (TFPI), protease nexin-1and protein S. It was shown that approximately 62% of plasma free TFPIα are stored in platelets, and TFPIα released from platelets was able to diminish plasma TG [60]. In addition, activated platelets induce APC resistance in TG testing via the release of platelet factor 4 (PF4) [61] and a special pool of FV(a) [62] from their α-granules. Furthermore, several independent groups have shown that platelets store in their α-granules considerable amount of TF [10] that are originated from megakaryocytes [63] or by de novo synthesis using mRNA templates [64]. Both the antigen and the procoagulant activity of TF have been detected on platelets [8, 65], although Østerud *et al.* and Bouchard *et al.* contradicted these findings [66-69], likely due to methodological differences [70, 71].

Platelet granular content have been shown to influence TG. An illustrating example was found in FV-deficient patients who have no detectable plasma FV and PPP-TG, in which the absence of life-threatening bleedings could be explained by FV-contribution from platelets leading to sufficient PRP-TG [72]. In addition, in patients with Quebec platelet disorder (QPD), who have defects in α-granule proteins including FV due to the presence of excessive α-granule urokinase-type plasminogen activator levels, normal PPP-TG profile but defective PRP-TG were found, and the lower ETP and peak thrombin in PRP showed a strong association with platelet FV level [73], further supporting a notable impact of platelet derived FV on TG.

Influence of platelet-coagulation factor binding on PRP-TG

Besides phosphatidylserine, several platelet membrane receptors can also bind coagulation factors and localize coagulation factors to the procoagulant surface, thus modifying the kinetics of TG [54]. For example, Glycoprotein (GP) Ib-V-IX binds VWF and brings VWF-bound FVIII to the platelet membrane. Integrin $\alpha_{IIb}\beta_3$, the most abundant receptor on platelets, binds fibrinogen and VWF in its active conformation. Furthermore, FXI is a ligand for Apolipoprotein E Receptor 2 (ApoER2) on platelets and thrombin is recognized by both GP Ib-V-IX and

protease activated receptors (PARs). There is also functional evidence suggesting the existence of receptors for FVIII, FIX and FX on the platelet surface [54].

The binding of coagulation factors to platelets can modify TG. Platelet inhibition *in vitro* by either blocking membrane receptors (e.g. $\alpha_{IIb}\beta_3$ blockage by abiciximab) or by blocking procoagulant surface (e.g. Annexin V) lead to decreased peak and ETP of PRP-TG [74, 75]. Glanzmann's thrombasthenia or Bernard Soulier syndrome, bleeding disorders due to deficiencies in platelet $\alpha_{IIb}\beta_3$ or GPIbα respectively, reduce the amount of thrombin generated in PRP [55, 76, 77]. Further evidence for a regulating role of platelets in TG are findings that the bleeding phenotype in patients with FXI deficiency can be better differentiated by measuring TG in PRP than PPP-TG testing [78, 79]. This observation implies the importance of physiological-relevant interactions between platelets and FXI during the amplification phase of TG, and suggests that PRP-TG could be a potential diagnostical tool in this setting.

Influence of platelet-VWF binding on PRP-TG

In primary hemostasis, VWF is an essential mediator for platelet adhesion and aggregation. Normally, VWF circulate in an inactive coiled conformation, but upon exposure to subendothelial collagen VWF expose its A1 domain and is able to bind platelet GPIb, which subsequently induces platelet activation [80]. Pelkmans *et al.* [81] showed that TG in the presence of platelets is sensitive to the activation status of VWF. They reported that a recombinant VWF variant (VWF-2B), which has a gain-of-function mutation in the VWF A1 domain that corresponds to mutations in type IIB VWD, was able to augment PRP-TG presumably because VWF-2B induced spontaneous platelet activation *via* GPIb. In addition, when TG was tested in reconstituted PRP by adding washed normal platelets resuspended in PPP from type 2B VWD patients or controls, the ratio of ETP in PRP/PPP was higher with patient plasma than that of controls, demonstrating that the procoagulant effect of VWF-2B is platelet-dependent.

VWF also mediates the translocation of FVIII from solution to platelet surface and therefore modulates TG on platelet surface. Patients with quantitative or functional defects in VWF have an increased risk of bleeding but this phenotype is not always explained by the degree of VWF deficiency [82]. Rugeri *et al.* studied TG in a group of patients with various types of von Willebrand disease (VWD) and found that these patients had markedly reduced ETPs and peak heights in PPP [83]. Interestingly, in PRP-TG they found comparable ETPs with a significantly decreased peak heights. The low FVIII level in these patients seemed to be the major cause of defect TG peak height, because FVIII supplementation was able to restore TG in both PPP and PRP, while VWF supplementation in the presence of normal FVIII level did not influence TG. Recently, Szanto and colleagues reproduced the above results in a group of patients with type 3 VWD (absence of VWF) in which they found diminished PPP-TG (lower peak and ETP) but overall comparable ETP and 13% decreased peak height in PRP-TG compared with healthy controls [84]. The reason behind the discrepancy between ETP and peak height of PRP-TG in VWD patients is still unclear but Szanto *et al.* [84] attribute the normal ETP in type 3 VWD to the compensatory role played by hyperresponsive platelets. Additional evidences are required to establish whether ETP or peak height of PRP-TG is more useful in predicting the bleeding risk

in these patients but Rugeri *et al.*[83] found that a subthreshold peak height was associated with a higher bleeding score in these patients, and argued that the ETP is not the only important parameter of the thrombogram.

Influence of platelet-autoantibody binding on PRP-TG

Autoantibodies against platelets can be generated due to immune disorders (immune thrombocytopenic purpura or systemic lupus erythematosus) or secondary to viral infections, as well as upon vaccination or administration of certain drugs [85]. Heparin-induced thrombocytopenia (HIT) is a rare but severe complication caused by autoantibodies against heparin-PF4 complex during heparin therapy and these antibodies cause spontaneous activation and accelerated clearance of platelets. Tardy-Poncet *et al.* [86] recapitulated the hypercoagulable state in these patients by showing that HIT antibody-positive PPP reacts stronger (higher peak height) to suppletion of unfractionated heparin than HIT-negative PPP after the plasmas were mixed with PRP of normal controls, thus demonstrating that HIT antibodies were able to potentiate platelet-dependent TG. Remarkably, they also showed that a HIT thrombogram profile defined by three ratios with /without heparin of TG parameters (peak, VI and time to peak) allowed sensitive identification (22 out of 23) of HIT-positive patients without false-positive, suggesting PRP-TG might have a place in the diagnosis of HIT.

Influence of platelet number and size on PRP-TG

Normal platelet count in humans is between 150 to 450 $*10^9$/L and altered numbers have been seen in thrombocytopenia and thrombocythemia. Studies on the influence of platelet counts on PRP-TG using reconstituted PRPs have shown that platelet counts correlate with TG acceleration if the platelet numbers are below 100 $*10^9$/L: increasing platelet counts reduce the lag time of TG and increase the peak thrombin level and ETP [32, 74, 87]. If the platelet numbers are above 100 $*10^9$/L, then platelet numbers still correlate with the peak height but the ETP becomes independent of the numbers. Currently, to control the pre-analytical variation of platelet count introduced during PRP preparation, platelet-dependent TG is often tested with platelet count adjusted to 150 $*10^9$/L [51], but this may mask the impact of individual platelet count differences, especially in patients with thrombocytopenia.

In the population-based Gutenberg Health Study which studied PRP-TG profile of approximately 400 individuals, mean platelet volume (MPV, a measure of platelet size) and platelet count were both independent determinants of PRP-TG parameters [88]. Increased platelet count and MPV were both related to shorter lag time and increased peak height. Platelet count was also significantly associated with increased ETP. Consistent with the above observation, increased MPV was related to higher platelet reactivity and was shown to be predictive of stroke and atrial fibrillation [89]. It was also shown that larger platelets (MPV ~11 fL) express three-fold higher levels of TF compared with smaller conterparts (MPV ~7 fL) [70].

Thrombocytopenia is a common complication of liver cirrhosis, with a prevalence of thrombocytopenia between 15 and 75 % [90]. Tripodi *et al.* studied thrombomodulin-modified TG with different counts of platelets in cirrhotic patients and found that the ETP of these

patients was lower than controls if platelet counts were adjusted to their whole blood level, but became comparable to normal controls if platelet count was adjusted to 100 $*10^9$/L [91]. They also showed that ETP was positively correlated with platelet counts and estimated that a minimum of 56 $*10^9$/L platelet is required to guarantee sufficient TG.

PRP-TG assays: advantages and limitations

PRP-TG is of additional value to PPP-TG because it gives insight in the role of platelets in coagulation and in the interplay between platelets and coagulation (summarized in Table 1 & 2). This may give insight in coagulation complications in platelet-related disorders.

Similar to PPP-TG, PRP-TG is performed under near-static conditions, so the impact of blood flow on the interaction between platelets and VWF or other coagulation protein is lacking. Furthermore, although the addition of thrombomodulin can partly resemble the anticoagulant function of endothelial cells, they also synthesize and release other coagulation proteins, most notably TFPI and VWF. The representation of endothelial function is also complexed by the fact that these proteins/receptors are differentially expressed across different vascular beds and are altered post activation [92].

It is also noteworthy that PRP-TG still needs further standardization to be clinically applicable. Although the existence of commercial assay systems [32] and expert recommendations [48, 51] ensures acceptable within-lab reproducibility, it may still suffer from large inter-center variations because of the lack of standardization of pre-analytical conditions between laboratories [93]. Because platelets are activated during long time storage, currently there is no established standardized PRP sample for PRP-TG results normalization [94]. As a result, comparison of results between different centers is still troublesome. The requirement that PRP needs to be tested within a short time (6 hours if blood is collected with corn tripsin inhibitor and 2 hours if not [49]) also raises logistic issues for large scale multicenter studies, which are currently needed to evaluate the real clinical value of this technique.

Table 2. Platelet-dependent thrombin generation tested in humans

Author (ref)	Tested population and characteristics	TG method	PPP-TG result	PRP-TG result	Platelet-related mechanism
Duckers [72]	Patients with severe congenital factor V deficiency	CAT; various TF concentrations & 20μM PL (PPP-TG); various TF concentrations (PRP-TG)	Lower or even undetectable TG in patients compared with controls	Adequate TG when platelets were pre-activated	Residual platelet FV and low TFPI level together ensured an adequate TG
Brunet [73]	Patients with Quebec platelet disorder (increased uPA in platelets)	CAT; 5pM TF & 4μM PL (PPP-TG); 0.5pM TF (PRP-TG)	Comparable ETP & peak height between patients and controls	↓ETP and peak height in patients	Decreased platelet α granule FV, due to exceptionally high kallikrein activity in platelets
Beguin [77]	Patients with Bernard-Soulier Syndrome (GPIb deficiency)	CAT; 0.5pM TF	None	↓ETP and peak height in patients compared with control	GPIb deficiency or inhibition (in normals) reduces TG in a fibrin-dependent manner
Reverter [55]	Patients with Glanzmann's thrombasthenia (integrin αIIbβ3 deficiency)	Subsampling; unknown TF; platelets of Glanzmann's thrombasthenia resuspended in normal PPP	None	↓ETP and peak height with patient platelets compared with normal platelets	αIIbβ3 deficiency or inhibition (in normals) reduces TG
Pelkmans [81]	Patients with type IIB Von Willebrand disease (VWF-2B variant, a gain-of-function mutation in the VWF A1 domain)	CAT; 1 or 5pM TF (PRP-TG)	Tested, but exact numbers and comparison between patients and controls not shown	↑ETP &peak after adding VWF-2B into normal PRP; normal platelets added into patient plasma had ↑ TG than added into normal plasma	Incubation of platelets with VWF-2B resulted in a 5-fold increased exposure of phosphatidylserine and a 3-fold increased expression of P-selectin
Rugeri [83] & Szanto [84]	Patients with von Willebrand's disease (type 1, 2 and/or 3)	CAT; 1pM TF & 4μM PL (PPP-TG); 0.5pM TF (PRP-TG)	↓ETP and peak height in patients compared with control	↓peak height, but comparable ETP	Enhanced platelet activation markers in flow cytometric assay
Tardy-Poncet [86]	Patients with heparin induced thrombocytopenia (HIT; autoantibody against PF4-heparin complex)	CAT; TF concentration unclear & ± 0.2U/mL UFH (PRP-TG); mixing patient PPP with PRP from normals	None	↑ratio of ETP, peak height & VI (with/without heparin) in HIT-positive than negative plasma	HIT antibody induced platelet activation, which could be abolished by blocking FcγRIIa receptor

Panova-Noeva [88]	407 adults from the population-based Gutenberg Health Study	CAT; 1pM TF & 4μM PL (PPP-TG); 1pM TF (PRP-TG)	No comparison	No comparison	Platelet count and MPV are independent determinants of ETP and peak height of PRP-TG in multivariable linear regression analysis
Tripodi [91]	Cirrhotic patients (reduced pro- and anticoagulant factors and thrombocytopenia)	CAT; 1pM TF, 1μM PL & 4nM TM (PPP-TG); 1pM TF & 4nM TM (PRP-TG)	Comparable ETP between patients and healthy controls; peak height not shown	↓ETP when PRP has physiological platelet count; but normal ETP when platelet count was adjusted to 100 *109/L	Platelet count of 56 *109/L is needed to guarantee sufficient TG

Abbreviations: TG, thrombin generation; PPP, platelet poor plasma; PRP, platelet rich plasma; CAT, calibrated automated thrombogram; TF, tissue factor; PL, phospholipids; ETP, endogenous thrombin potential; FV, factor V; TM, thrombomodulin; HIT, heparin induced thrombocytopenia; PF4, platelet factor 4; UFH, unfractionated heparin.

WB-TGAS AND THE INFLUENCE OF ERYTHROCYTES, LEUKOCYTES AND CANCER CELLS

In contrast to platelets whose role in coagulation has been well-established, less is known about the involvement of other circulating blood cells in coagulation and TG. WB-TGA is a promising solution to study the interplay between all blood cells and coagulation in relation to thrombotic and bleeding complications. There are some studies about the influence of erythrocytes on TG, but other blood cells such as leukocytes, cancer cells remain largely unexplored.

WB-TG assays: technical challenges and recent advances

Although TG with the subsampling technique can be applied to all blood preparations including WB, it has major drawbacks: it is time- and labor-consuming. Furthermore, this method only measures active thrombin in free solution but not those bound to fibrin [32]. Alternatively, an ELISA based assay was developed to quantify the thrombin-antithrombin (TAT) complex in timed subsamples from clotting WB [95]. This assay allows indirect estimation of TG but is still time-consuming. An electrochemical TGA for plasma and WB has also been introduced in 2009 [96], but was not reproduced in any further studies. Continuous chromogenic TG assays cannot be applied in WB because erythrocytes seriously disturb optical measurement.

Fluorogenic TG assays allow continuous and high throughput TG measurements in plasma, but their application in WB has been challenging. Erythrocytes sediment and contract with clot during TG measurement, which may cause variable quenching of the fluorescent signal over time and lead to erratic signals [97, 98]. Several methods have been previously presented to solve this problem. In 2007, Tappenden *et al.* [97] reported a method in which an orbital shake was

applied to the assay plate during the idle time between two rounds of readings, but this method still gave rather high variations. Ninivaggi *et al.* [99] later reported another approach which utilizes a filter paper matrix to restrain the erythrocytes in a thin layer to avoid erythrocyte sedimentation. This technique yields reproducible results, but requires good level of pipetting to guarantee reproducibility [100]. In 2016, Kelchtermans *et al.* reported a rheometer-based fluorogenic assay for simultaneous measurement of TG and fibrin formation in PPP and WB [30], in which the issue of erythrocyte sedimentation was minimized by constantly mixing the blood with the rotation of the cone in the rheometer. A major disadvantage of this procedure is the low sample throughput: only one sample can be measured per run. Recently we developed a novel fluorogenic TG assay for WB measurements in which erythrocyte sedimentation is prevented by the continuous mixing induced by maintaining a continuous movement of the assay plate [87]. This assay has good reproducibility, meanwhile requiring less handling than the filter paper-based assay.

Influence of erythrocytes phosphatidylserine and number on WB-TG

Until recently, erythrocytes have been considered as passive bystanders in coagulation and are often omitted in coagulation tests. However, clinical abnormalities in both erythrocyte quantity (elevated hematocrit) and quality (e.g. sickle cell disease, thalassemia) have been associated with arterial and venous thrombosis [101-105]. Causality of this relation has not been determined, although it was speculated that altered blood rheology, caused by the elevated hematocrit, altered erythrocyte deformability and the formation of erythrocyte aggregates (rouleaux), could reduce the velocity of blood flow, diminish the anti-adhesive effect of endothelium derived nitro oxide (NO) and increases the migration/adhesion of platelets to the vessel walls [101, 102, 106, 107]. Apart from the above mentioned mechanism, a subset of normal erythrocytes [108] and erythrocyte-derived microparticles (MPs) showed phosphatidylserine exposure [106], and patients with certain diseases (e.g. sickle cell disease) had an up to 10 times higher proportion of phosphatidylserine-positive erythrocytes compared to healthy individuals [109]. Considering that erythrocytes are more abundant (around 20 times higher) and have a larger size than platelets (6-10 vs 1.5-3 μm in diameter, respectively), they may represent a major source of procoagulant phosphatidylserine in a red clot.

WB-TG is dependent on erythrocyte count in reconstituted blood. Using the subsampling technique, Peyrou *et al.* and Horne *et al.* showed that peak thrombin increased when the erythrocyte count added into PPP increased from 0 to a physiological level (hematocrit = 40%), similar to the effect seen when adding platelets into PPP [108, 110]. The ability of erythrocyte to support TG could be related to their membrane phosphatidylserine exposure [108]. Similar observations were also obtained using fluorogenic methods [87, 99, 111]. Moreover, we recently showed that erythrocytes augmented peak thrombin level even in the presence of high platelet count [87]. Furthermore, both in a group of healthy adults [87] and cirrhotic patients [112] we observed a positive correlation between hematocrit and the peak of WB-TG, indicating that erythrocytes have a positive effect on the velocity of WB-TG.

Interestingly, erythrocyte count exhibits a different effect on the ETP than peak height in WB-TG [87]. In reconstituted blood ETP was augmented when the hematocrit was increased from 0 to 20%, but it remained largely unchanged when the hematocrit further increased from 20 to 45%. In contrast, the peak of WB-TG was affected in the full range of hematocrit from 0 to 45%. The differential influence of erythrocyte counts on peak and ETP was also observed in the WB-TG profiles of cirrhotic patients [112]. In cirrhotic patients, erythrocyte and platelet counts are only moderately reduced, however this reduction in hematocrit decreased peak thrombin level but not the ETP level of WB-TG [112].

Studies on the influence of erythrocyte (dys)function on WB-TG in disease settings are scarce. Sickle cell disease (SCD) results in elevated plasma TAT and D-dimer levels, and is associated with an increased thrombosis risk [105, 113]. In contrast to *in vivo* data, *in vitro* tests in plasma of SCD patients showed that this procoagulant phenotype was not due to plasma factors, as the PPP-TG in SCD patients was comparable or lower than in healthy controls. In whole blood, it was shown that SCD patients form more TAT complexes in TF trigged WB [113], thus indicating that sickled erythrocytes cause the hypercoagulation in SCD patients. A higher percentage of the erythrocytes from SCD were phosphatidylserine-positive but, interestingly, the percentage of phosphatidylserine exposure was negatively correlated with WB-TAT formation, suggesting certain cellular factors, other than phosphatidylserine exposure, contribute to TG in SCD. Alternatively, erythrocyte membrane and erythrocyte-derived MPs can trigger coagulation through the intrinsic pathway [114-116]. Recently, it is shown that an unrecognized protein in erythrocyte MPs directly activated FXII and prekallikrein [117]. The activation of the contact pathway not only induce FXI activation; it was also shown that FXIIa and kallikrein can both directly activate FIX [117-119].

Influence of erythrocyte-platelet interaction on WB-TG

Platelets and erythrocytes are the most abundant cells in circulating blood and their interactions may be an underestimated player in coagulation. Erythrocytes can directly activate platelets through the release of ATP and ADP [120]. Additionally, intercellular adhesion molecule (ICAM)-4 on erythrocytes was suggested as ligand for $\alpha_{IIb}\beta_3$ on platelets [121], and their interaction was shown to further promote platelet activation as evidenced by increased p-selectin expression. In accordance, the blockage of ICAM4-$\alpha_{IIb}\beta_3$ interaction caused reduced fibrin and thrombus formation in an *in vitro* perfusion model [106]. An alternative direct interaction between erythrocytes and platelets is mediated by binding of Fas receptor (FasR) on erythrocytes to Fas ligand (FasL) on activated platelets, which promotes phosphatidylserine exposure on both cells [122]. Blockage of this interaction leads to reduced thrombus formation under flow, reduced phosphatidylserine exposure on platelets and erythrocytes, as well as reduced WB-TG. FasR- or FasL-knockout mice exhibit delayed initiation of thrombus formation and reduced occlusion after $FeCl_3$ treatment on mesenteric arterioles [122]. Interfering with the FasL-FasR interaction might be an innovative and promising approach for a completely novel antithrombotic strategy.

Influence of leukocytes on TG

Leukocytes are important players in host defense and regulation of inflammatory response. Leukocytes are categorized into neutrophils (constitute 62% of all leukocytes), eosinophils (2.3%), basophils (0.4%), lymphocytes (30%), and monocytes (5.3%). Monocytes have been known to synthesize and express TF on their cytoplasmic membrane and release TF-positive MPs in response to various cytokines, growth factors and biogenic amines [9, 123]. Although lipopolysaccharides (LPS) stimulated monocytes also express TFPI besides TF, it was still able to induce considerable amount of TG, which was only marginally enhanced by anti-TFPI antibodies [124]. Synthesis of TF in neutrophils is controversial [125-127], but neutrophils can release procoagulant matrix metalloproteinases and serine proteases, such as cathepsin G and elastase upon stimulation and directly activate FV, FVIII and FX, as well as downregulate anticoagulant factors including antithrombin, heparin cofactor II and TFPI [128]. In addition, stimulated neutrophils release chromatin components, i.e. neutrophil extracellular traps (NETs), after exposure to microorganisms, inflammatory cytokines and activated platelets [128, 129]. NETs serve as a scaffold for many procoagulant stimuli, such as platelets, erythrocytes, VWF and TF [130]. Moreover, several components of NETs have been independently shown to trigger coagulation. For example, histone H4 directly triggers autoactivation of prothrombin to thrombin [131], whilst contact pathway activation can be triggered by purified DNA, albeit different purification methods yeilds DNA products with drastically different procoagulant activities [132, 133]. Furthermore, activated leukocytes induce platelet activation and aggregation by granule release and ligand-receptor interactions [134].

Influence of cancer cells on TG

Patients with cancer have a 5-fold higher risk of venous thromboembolism than those without malignancy [135-137]. The mechanism of cancer-associated hypercoagulability is still unclear and is not always detectable with PPP-TG [138, 139]. Several types of cancer cell lines, including pancreat, leukemia and breast origins, express TF, and these cells can induce coagulation in CAT assays [6, 7, 140]. Moreover, cancer cell-induced TG is inhibited by anti-TF antibodies, suggesting a role for *in vivo* TF-positive cancer cells in thrombogenesis [6, 7, 140], although the expression of TF on cancer cells and its effect on TG varies between different cancer types [138]. Procoagulant MPs have been found in both cultured cancer cell lines [141-143] and in the circulation of cancer patients [143-145] and these vesicles were found to support TG, probably because of TF or phosphatidylserine exposed on their outer membrane. Furthermore, malignant tissues have been reported to produce a cysteine protease that triggers coagulation by directly activating FX independent of FVIIa [146, 147]. Cancer cells also promote coagulation activation indirectly through the activation of other blood cells; tumour-derived factors, such as ADP, thrombin and cytokines can activate platelets and/or leucocytes, resulting in phosphatidylserine-positive platelet surfaces or NETs; both are known to be procoagulant (reviewed in [138, 148]).

Although the influence of leukocytes, cancer cells and MPs on TG has been studied in isolated plasma model system, their influence in a complex near-physiological environment like WB remains to be assessed. Further studies are needed to advance this field.

WB-TG assays: advantages and limitations

Compared with plasma tests, WB-TG assays do not need the centrifugation steps for plasma preparation, thus avoid the possible pre-analytical variations of plasma preparation and allow faster measurement. By including all circulating cells, WB-TG reaches one step closer to physiology than plasma coagulation tests and maybe useful for the assessment of blood cells-related coagulation disorders (Table 1 and Fig. 2). In addition, WB-TG is inhibited by antiplatelet drugs [87] and anticoagulants (rivaroxaban and dabigatran, unpublished data), suggesting a potential of these assays to serve as a point-of-care (POC) test to monitor anticoagulant and antiplatelet treatments.

The major difference that WB-TG assays have over the currently established POC viscoelastic tests (rotational thromboelastometry [ROTEM] or thrombelastography [TEG]) is that the latter measures the elasticity of a blood clot instead of the amidolytic activity of thrombin [149]. The viscoelastic tests have shown usefulness for the management of bleeding complications during surgery and in acute trauma care, especially in reducing the amount of blood product transfusion [150]. However, assessing fibrin clot formation only reflects the procoagulant effect of thrombin, whereas incomplete information is given on the anticoagulant pathways, particularly the anticoagulant effect of thrombin through the protein C pathway. On the contrary, WB-TG covers the entire course of thrombin activation and inactivation, even including the function of the protein C pathway if thrombomodulin is supplemented, making it more suitable for predicting thrombophilia [87, 112].

WB-TGA is still in its infancy and a long way from clinical application. The lack of standardization on pre-analytical variables and analytical protocols, as well as the absence of standardised reagents and reference sample greatly hampers the wide evaluation of its clinical application. For example, the use of CTI was shown to improve the reproducibility of plasma-TG when the assay was triggered with low concentration of TF [48, 51]. WB-TG is typically triggered with low amounts of TF (0.5 or 1 pM) [87, 99] so that coagulation is dependent on physiological PL provided by blood cells and on feedback loops, but whether the use of CTI would also impact the reproducibility of the WB-TG assay requires further assessment. The assay trigger should also be optimized for different settings; for instance a trigger that contain low or even no TF maybe necessary to reflect the procoagulant effect of TF/phosphatidylserine on cancer cells or microparticles. Lastly, similar to PRP-TGA, the influence of blood flow and endothelium is not represented, and it is a technical challenge to develop a standard sample for result normalization.

GENERAL DISCUSSION ON THE ADDED VALUE OF PRP-TG AND WB-TG

Blood cells are important players in coagulation and can influence TG testing. The complex interplay between platelets and the coagulation system has been underappreciated for many decades. Physiological platelet thrombus formation is augmented by thrombin formation, while physiological coagulation is in many stages facilitated by expression of procoagulant surfaces, the expression or activation of specific receptors on platelets and the delivery of FV. As shown above, the differentiation of platelet related-coagulation disorders, including platelet secretion/receptor defect, VWD and FXI-related bleeding disorder requires the presence of platelets to ensure a more comprehensive TG profiling. In addition, abnormally high hematocrit, TF-positive cancer cells, inflammation-activated monocytes have all been shown to promote TG by exposing phosphatidylserine and/or TF, therefore testing WB-TG is a more logical choice than PPP-TG in this context. Furthermore, there are active interaction between blood cells, such as between platelets and erythrocytes or between platelets and leukocytes. The effect of these interactions on TG need to be assessed in a WB context. Our group recently developed a WB-TG assay that shows good correlations with plasma TG tests, with platelet/erythrocyte numbers and with the use of platelet inhibitors [87], and may serve as a useful tool for studying the involvement of blood cells in coagulation.

There are also limitations with these blood cells-related TGAs. Firstly, these is a lack of standardization. The inter-laboratory variation of PPP-TG has been drastically improved by ensuring good thermal control, the use of standardized pre-analytical protocols and reagents, as well as the use of normal pooled plasma to normalize the data in each run [48-52]. In contrast, the standardization of pre-analytical conditions and analytical variables for PRP- and WB-TG are still poor [93]. Furthermore, due to the limitation that PRP or WB cannot be stored over long time, there is still no standardized sample for the normalization of the TG parameters in PRP and WB, this may further hamper result comparison between different centers. Secondly, it is noteworthy that, similar to PPP-TG, some aspects of physiological coagulation are not represented in PRP- or WB-TGAs, including the effect of blood flow and endothelium, although the latter can be partly represented by adding soluble thrombomodulin. Lastly, the differential effect of blood cell counts on the TG parameters, most notably peak and ETP, of PRP- and WB-TG calls for caution when interpreting the results of these assays. Further mechanistic and clinical studies are needed to determine which parameter(s), or the integration of parameters into a score, are most useful.

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CHAPTER 6

Plasmatic coagulation capacity correlates with inflammation and abacavir-use during chronic HIV-infection

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ABSTRACT

Background

D-dimer concentrations in people living with HIV (PLHIV) on antiretroviral therapy (cART) are increased and have been linked to mortality. D-dimer is a biomarker of *in vivo* coagulation, In contrast to reports on D-dimer, data on coagulation capacity in PLHIV are conflicting. Here, we assessed the effect of cART and inflammation on coagulation capacity.

Setting

We explored coagulation capacity using *calibrated* thrombin generation (TG) and linked this to persistent inflammation and cART in a cross-sectional study including PLHIV with viral suppression and uninfected controls.

Methods

We used multivariate analyses to identify independent factors influencing *in vivo* coagulation (D-dimer) and *ex vivo* coagulation capacity (TG).

Results

Among 208 PLHIV, 94 (45%) were on an abacavir containing regimen. D-dimer levels (219.1 vs 170.5 ng/mL, p=0.001) and inflammatory makers (sCD14, sCD163 and hsCRP) were increased in PLHIV compared to controls (n=56). PLHIV experienced lower thrombin generation (reflected by endogenous thrombin potential; ETP) compared to controls, after correction for age, sex and antiretroviral therapy. Abacavir-use was independently associated with increased ETP. Prothrombin concentrations were strongly associated with ETP and were lower in PLHIV on a non-abacavir containing regimen compared to controls, suggesting consumption as a possible mechanism for HIV-associated reduction in TG. D-dimer concentrations were associated with inflammation, but not TG.

Conclusion

Abacavir-use was associated with increased TG and could serve as an additional factor in the reported increase in thrombotic events during abacavir use. Increased exposure to triggers that propagate coagulation, such as inflammation, likely underlie increased D-dimer concentrations found in most PLHIV.

Key words: D-dimer, thrombin generation, abacavir, inflammation, coagulation, thrombosis

INTRODUCTION

Successful combination antiretroviral treatment (cART) has nearly normalized life expectancy of people living with HIV (PLHIV), although treatment is not able to fully reverse immune activation and persistent inflammation[1-3]. This inflammation may increase the risk for thrombotic events [4]. While increased risk for venous thrombosis PLHIV appears to be limited to those with incomplete CD4 recovery or with continuing viral replication [5, 6], data on arterial thrombosis risk show an increased cardiovascular risk in well-treated PLHIV [7-9]. There is an ongoing debate on the effect of cART, especially abacavir, on cardiovascular risk [10-15].

D-dimers, which are soluble fibrin degradation products, are a marker of coagulation activity in the body. HIV infection is associated with elevated D-dimer concentrations even in those with viral suppression [16-18]. D-dimer concentrations are independently associated with overall mortality and the incidence of cardiovascular disease and cancer in PLHIV [9, 16, 19]. D-dimer concentrations are influenced by activation of the coagulation cascade itself, as well as signals that provoke coagulation such as inflammation and endothelial activation [4]. Indeed, the inflammatory markers sCD14, high-sensitive C-reactive protein (hsCRP) and sCD163 have been associated with D-dimer in PLHIV [17, 20], suggesting a link between inflammation and *in vivo* coagulation activity in PLHIV.

The capacity of plasma to form thrombin is a critical determinant of in vivo plasmatic coagulation [21]. Thrombin generation (TG) can be measured *ex vivo* to determine coagulation capacity in a standardized setting and has been used as a diagnostic tool for hypo- and hypercoagulability states [22]. In contrast to elevated D-dimer concentrations in PLHIV [16-18], available data on *ex vivo* TG in PLHIV are contradictory [23-25]. This also applies to the possible roles of inflammation and cART on TG.

Hence, we measured TG, as well as different coagulation markers, in a cross-sectional cohort of cART treated, virally suppressed PLHIV and HIV-uninfected controls and related TG to markers of inflammation and cART. We hypothesized that TG is influenced by both persistent inflammation and cART.

MATERIALS AND METHODS

Study procedures

This cross-sectional, prospective study was performed at the Radboud university medical center, a tertiary teaching hospital in The Netherlands. The study was conducted in accordance with the Declaration of Helsinki after approval of the ethics committee (CMO Arnhem-Nijmegen, The Netherlands; NL42561.091.12, 2012/550). This study was embedded in the Human Functional Genomics Project (HFGP; www.humanfunctionalgenomicsproject.org). Adult HIV-1 infected individuals and controls were concurrently enrolled after a written informed consent

was obtained. Inclusion criteria included suppressed viral load (<200copies/mL) after cART use for at least six months. Exclusion criteria were the use of coumarin derivates or direct anticoagulant therapy, as well as active hepatitis B or C infection, and/or signs of an infection other than HIV-1.

Plasma thrombin generation measured with and without thrombomodulin

TG in platelet poor plasma (PPP) was measured with the "MidiCAT" technique, which is a modified calibrated thrombin generation (CAT) for the measurement of samples with low plasma volume. [22, 26] This technique maintains the plasma dilution ratio while requiring only half of the volume (i.e. 40 versus 80 µL per well) needed than the regular CAT technique. PPP was stored at -80°C until thawed at 37°C for 10 min before measurement. TG was triggered with 5 pM tissue factor (TF; Innovin, Seimens Healthcare Diagnostics, Marburg, Germany), 4 µM phospholipids (PL; Avanti Polar Lipids Inc., Alabaster, AL, USA) and in the presence and absence of 7 nM thrombomodulin (TM; Synapse research institute, Maastricht the Netherlands). The concentration of TM was chosen to inhibit the ETP by 50% in normal pooled plasma (NPP). TG parameters were calculated using specialized software from Thrombinoscope B.V. (Maastricht, the Netherlands). TG parameters (supplemental figure 1) including Lagtime (LT; min), time-to-peak (TTP; min), Peak (nM), endogenous thrombin potential (ETP; nM×min) and velocity index (VI; nM/min) were chosen for further analysis. The ETP, Peak and VI of tested subjects were normalized as the percentage of that of normal pooled plasma (NPP) tested without TM in the same run.[26]

The sensitivity of the TG parameters to TM reflects the function of the anticoagulant protein C pathway. The TM sensitivity ratio of ETP (ETP-TMsr) was calculated as the ratio of the ETP in the presence of TM and ETP in the absence of TM. The normalized TM sensitivity ratio of ETP (nETP-TMsr) was calculated by dividing the ETP-TMsr of subject by that of NPP in the same run. The nPeak-TMsr and nVI-TMsr were calculated similarly. A nTMsr value less than 1 means that the PPP of the tested subject have a better functioning protein C system than the NPP, and vice versa.

Plasma markers of coagulation and inflammation

Inflammatory markers sCD163 (Quantikine), sCD14 (Quantikine) and hsCRP (Duoset) were determined in EDTA plasma by ELISA (all R&D system, Minneapolis, USA). D-dimer was measured by ELISA according to manufacturer's instructions (Abcam, Cambridge, UK).

The measurement of plasmatic fibrinogen was performed using the Clauss-method on a Start4 analyzer (Dignostica Stago, Asnières, France) with a known fibrinogen reagent (Dade® Fibrinogen Determination Reagents, Siemens, Munich, Germany). Prothrombin (sheep anti-human prothrombin polyclonal antibody and HRP-conjugated sheep anti-human prothrombin polyclonal antibody; Affinity Biologicals, Ancaster, Canada), protein S (sheep anti-human protein S IgG antibody and HRP-conjugated sheep anti-human protein S antibody from Affinity Biologicals and von Willebrand factor (vWF; Rabbit anti-human vWF and HRP-conjugated rabbit anti human vWF; DAKO, Agilent, Santa Clara, USA) concentrations were performed

with an in-house sandwich ELISA assay. Briefly, 96 wells microtiter plates (NUNC Maxisorp, Thermo Fisher Scientific, Waltham, USA) were coated overnight at 4°C with capture antibody in a carbonate-bicarbonate coating buffer (pH 9.6) and blocked with 2% BSA in phosphate-buffered saline (PBS) for 45 minutes at room temperature (RT) before adding diluted plasma samples and incubated at RT for 1.5 hour. The wells were then incubated with in detection antibody in PBS/2%BSA for 2 hours at room temperature after washing. Plates were washed before addition of SIGMAFAST OPD (Sigma). After 30 minutes the reaction was stopped with 3 M sulfuric acid (H2SO4, Sigma). Optical densities (OD) were measured at 490 nm using an ELx808 Absorbance Microplate Reader (Biotek, Bad Friedrichshall, Germany). A calibration curve of serial diluted NPP was added to each plate [27, 28]. Consequently, the concentrations of prothrombin and protein S were expressed as the percentage (%) of the normal NPP.

Statistical analysis

R version 3.5.1 (CRAN-project) was used for analyses. Comparison between groups was done by Mann-Whitney U test, Student's T-test or Chi Square test depending on data distribution. The primary outcome for TG used in our analyses was ETP. Other TG parameters were treated as exploratory parameters. Benjamini-Hochberg procedure was performed on circulating markers and ETP comparisons (Figure 2, Table 2). For univariate and multivariate linear regression data of the dependent variable was transformed by log- or inverse rank transformation depending on distribution. All multivariate linear regression models include a parameter to correct for possible storage degradation or time of inclusion bias. The correlation matrix was performed using spearman's correlation coefficient. Missing data for all parameters was below <2.5% and comparisons were done pairwise.

RESULTS

A total of 208 virally suppressed PLHIV on stable cART and 56 uninfected controls were concurrently measured and included in the analysis. Baseline characteristics can be found in table 1. PLHIV were more often male (91.3% vs 60.7% p<0.001) and older (52 [46-59] vs 30 [26-53] years p<0.001) compared to HIV uninfected controls. An abacavir containing regimen was used by 94 PLHIV (45%) and 140 PLHIV (67%) used an integrase inhibitor (INSTI)-based regimen.

First, *in vivo* coagulation activity, determined by D-dimer, was increased in PLHIV compared to controls (219.1 vs 170.5 ng/mL respectively p=0.001; table 2), as were all markers of inflammation (hsCRP, sCD14, sCD163) and the microbial translocation marker IFABP (data shown in table 2). D-dimer was independently associated with age (B= 7.44, p<0.001) and after correction for age and sex, the difference in D-dimer between PLHIV and uninfected controls disappeared (B= 0.154, p=0.878;supplemental table 1). The markers of inflammation and microbial translocation remained significantly increased in PLHIV compared to controls after correction for age and sex (supplemental table 1). D-dimer concentrations correlated with markers of inflammation, IFABP, and endothelial activation (plasma vWF; see Figure 1).

Table 1 Baseline characteristics of PLWHIV and uninfected controls stratified by abacavir use.

	No ABC	ABC	HC	P-value (ABC vs no-ABC)	P-value (HC vs PLHIV)
N	114	94	56		
Sex (Female) (%)	13 (11.4)	5 (5.3)	22 (39.3)	0.192	<0.001
AGE (median [IQR])	53.0 [47.0, 60.0]	50.0 [41.5, 58.0]	30.0 [25.8, 53.0]	0.086	<0.001
BMI	24.2 [22.4, 26.0]	23.8 [21.8, 26.2]	23.8 [21.5, 25.6]	0.469	0.518
HIV infection (years)	10.0 [6.2, 16.8]	6.5 [4.3, 10.3]		<0.001	
Way of transmission (%)				0.245	
Heterosexual	3 (2.6)	5 (5.3)			
IDU	2 (1.8)	1 (1.1)			
MSM	82 (71.9)	75 (79.8)			
Other/unknown	27 (23.7)	13 (13.8)			
CD4 nadir	205.0 [120.0, 347.5]	275.0 [185.0, 377.5]		0.044	
CD4 count	645.0 [482.5, 827.5]	665.0 [492.5, 800.0]		0.754	
Viral load <40 copies/mL	111 (97.3)	94 (100%)		0.317	
CD4-CD8 ratio	0.8 [0.5, 1.1]	0.8 [0.6, 1.1]		0.954	
cART duration	7.9 [4.9, 15.4]	5.5 [3.7, 8.6]		<0.001	
NNRT (%)	44 (38.6)	17 (18.1)		0.002	
PI (%)	26 (22.8)	6 (6.4)		0.002	
INSTI (%)	67 (58.8)	73 (77.7)		0.006	
NRTI FTC (%)	91 (79.8)	0 (0.0)		<0.001	
NRTI 3TC (%)	14 (12.3)	91 (96.8)		<0.001	
NRTI AZT (%)	3 (2.6)	1 (1.1)		0.755	
NtRTI TDF (%)	92 (80.7)	1 (1.1)		<0.001	
Prior myocardial infarction (%)	6 (5.3)	4 (4.3)		0.99	
Prior stroke (%)	2 (1.8)	1 (1.1)		1	
VTE or pulmonary embolism (%)	3 (2.6)	1 (1.1)		0.755	
Smoking (%)	33 (28.9)	24 (25.5)		0.694	
Hypercholesterolemia (%)	33 (28.9)	23 (24.5)		0.57	
Hypertension (%)	23 (20.2)	15 (16.0)		0.546	
Diabetes Mellitus (%)	6 (5.3)	2 (2.1)		0.419	
Family history of CVD 1st degree (%)	59 (51.8)	44 (46.8)		0.568	
Cholesterol lowering drugs (%)	35 (30.7)	21 (22.3)		0.232	
Antihypertensive drugs (%)	27 (23.7)	20 (21.3)		0.805	
ASA (%)	10 (8.8)	8 (8.5)		1	

Abbreviations: ABC: abacavir; IDU: intravenous drug-use; MSM: man who have sex with man; HIVRNA blib: viral load of 40-500 copies/mL after previous viral suppression and direct subsequent suppressed viral load; NRTI: nucleoside reverse transcriptase inhibitor; NtRTI: nucleotide reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; INSTI: integrase inhibitor; FTC: emtricitabine; 3TC: lamivudine; AZT: zidovudine; TDF: Tenofovir difumarate; TAF: tenofovir alafenamide;

EFV: efavirenz; ETR: etravirine; NVP: nevirapine ; RPV: rilpivirine ; ATV: atazanavir; DRV: darunavir ; LPVr: lopinavir; IDV: indinavir; MVC: maraviroc; DTG: dolutegravir; EVG: elvitegravir; RAL: raltegravir; ASA: acetyl salicylic acid; CVD: cardiovascular disease. Data were displayed as number (%) or median [IQR], and were analyzed using either chi-square test or Mann-Whitney U-test depending on data distribution.

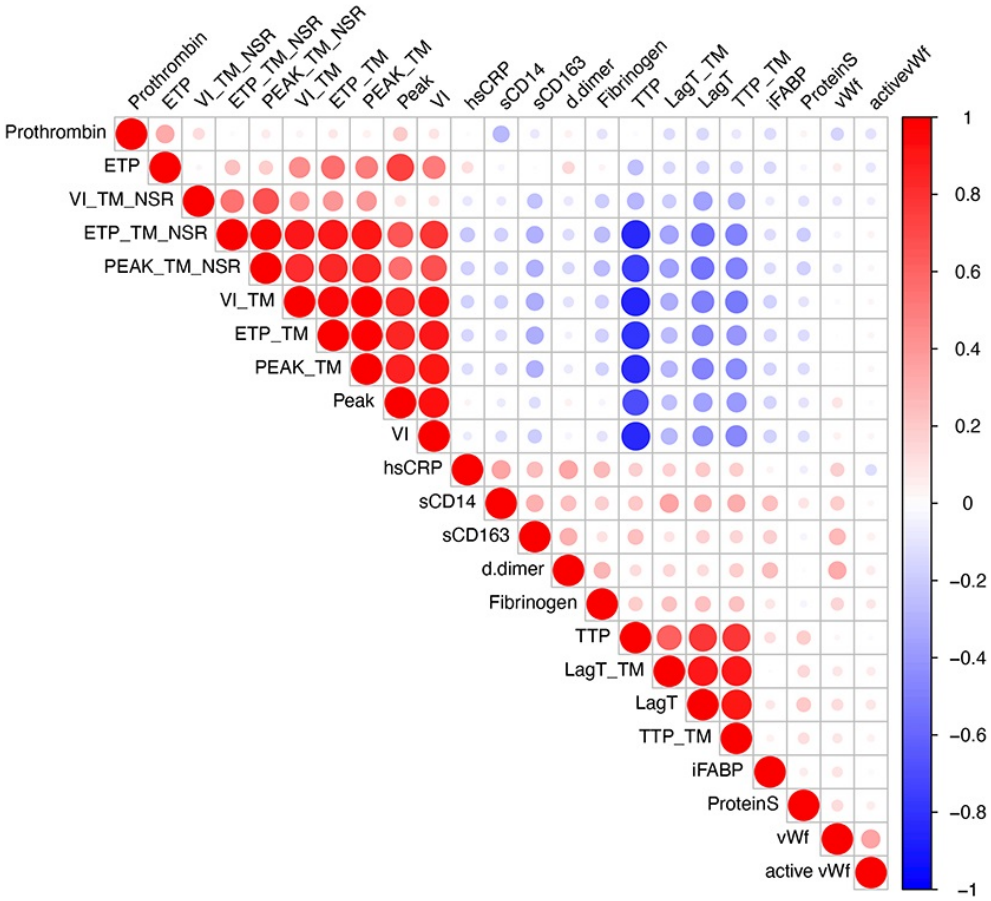

Figure 1 Spearman correlation matrix. Spearman's Correlation coefficients are shown in color and size of circles. Parameters are clustered using hierarchal clustering based on coefficients. Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Ex vivo coagulation capacity by thrombin generation

Next, *ex vivo* coagulation capacity was determined by *ex vivo* calibrated thrombin generation in plasma. Overall, calibrated thrombin generation showed lower thrombin generation, most notably reduced endogenous thrombin potential (ETP), our primary outcome regarding TG, in PLHIV compared to uninfected controls (table 2, FDR-corrected). In addition PLHIV experienced reduced peak thrombin formation and increased lag time PLHIV compared to HIV-negative controls (data shown in table 2). This difference in ETP persisted (FDR-corrected) only in a subgroup analysis of participants aged 40 years and above (Supplemental table 2), but disappeared in the subgroup of only men (supplemental table 3). However, after correction for age and sex, none of the thrombin generation parameters were significantly different between both cohorts (supplemental figure 1). Most women used oral contraceptive during sampling, which may account for the strong changes in TG parameters when stratifying for sex in the uninfected cohort [29]. Within the PLHIV cohort, no clear association was found between ETP and CD4 nadir, HIV duration or current CD4 count (Figure 1; supplemental table 5). Higher current CD4 count, and CD4-recovery were correlated with increased peak thrombin and ETP (data shown in supplemental table 5), whereas HIV-RNA zenith and HIV duration were associated with decreased protein C activity (nETP-TMsr; supplemental table 4). With the exception of nETP-TMsr, we found no associations between smoking and TG parameters (supplemental table 5).

Inflammation and ex vivo coagulation capacity

While inflammatory markers and D-dimer correlated positively, ETP did not correlate with any of the included inflammatory markers. Only lag time and time-to-peak correlated with sCD14, sCD163 and hsCRP (data shown in figure 1). Moreover, sCD163 showed a negative correlation with Protein C activity (nETP-TMsr; R -0.2, $P<0.05$, figure 1).

Table 2 Markers of inflammation and coagulation in vivo and in vitro.

	PLHIV	HC	P	PLHIV -no ABC	PLHIV - ABC	P
N	208	56		114	94	
D-dimer	219.1 [160.6, 334.8]	170.5 [122.0, 307.2]	0.002	213.4 [158.6, 335.5]	221.9 [162.6, 334.2]	0.673
Fibrinogen	3.4 [2.8, 4.0]	3.2 [2.8, 4.1]	0.746	3.4 [2.8, 4.0]	3.3 [2.8, 4.0]	0.632
Prothrombin	109.0 [89.3, 135.1]	135.1 [102.4, 162.5]	<0.001	106.0 [87.1, 135.1]	113.4 [94.8, 134.8]	0.305
Protein S	91.4 [80.7, 110.3]	86.9 [72.7, 112.4]	0.121	91.7 [81.7, 109.4]	91.4 [78.2, 110.4]	0.937
vWF	42.7 [31.8, 59.4]	32.5 [24.4, 43.9]	<0.001	42.3 [31.6, 61.6]	43.0 [32.4, 57.3]	0.623
iFABP	499.6 [263.0, 717.1]	242.5 [112.9, 376.9]	<0.001	576.2 [287.4, 897.2]	400.6 [240.5, 619.2]	0.004
hsCRP	1446 [608, 2735]	651 [205, 1179]	<0.001	1558 [624, 3285]	1192 [594, 2249]	0.337
sCD14	2139 [1778, 2625]	1789 [1502, 2071]	<0.001	2063 [1745, 2591]	2236 [1830, 2684]	0.193
sCD163	716.2 [525.6, 898.8]	517.3 [410.7, 578.1]	<0.001	765.8 [581.6, 916.1]	634.5 [487.8, 858.3]	0.019
PPP-TG						
Lag time	2.0 [1.7, 2.3]	2.0 [1.7, 2.0]	0.382	2.0 [1.7, 2.3]	2.0 [1.7, 2.3]	0.557
TTP	4.4 [4.0, 5.0]	4.3 [3.5, 5.0]	0.035	4.3 [4.0, 5.0]	4.4 [4.0, 5.0]	0.566
ETP	86.6 [78.6, 99.6]	93.0 [83.3, 105.0]	0.011	83.7 [74.7, 97.6]	90.2 [82.7, 101.6]	0.001
Peak	82.6 [70.0, 95.8]	88.1 [72.3, 101.5]	0.031	79.9 [65.2, 96.0]	85.8 [76.1, 95.7]	0.083
VI	78.1 [56.2, 102.7]	97.3 [64.0, 123.5]	0.001	76.4 [53.6, 102.5]	78.3 [61.2, 102.5]	0.566
LagT-TM	1.7 [1.4, 2.0]	1.7 [1.4, 2.0]	0.704	1.7 [1.4, 2.3]	1.7 [1.7, 2.0]	0.645
TTP-TM	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.641	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.982
ETP-TM	40.6 [27.9, 52.0]	50.1 [32.8, 76.2]	<0.001	41.0 [28.0, 51.1]	39.7 [26.3, 52.6]	0.706
PEAK-TM	49.6 [31.8, 62.4]	57.1 [39.8, 82.5]	0.002	50.9 [33.2, 61.6]	47.9 [29.9, 62.5]	0.553
VI-TM	59.8 [38.6, 82.5]	71.9 [51.8, 112.5]	<0.001	62.1 [39.8, 85.4]	59.6 [33.2, 77.5]	0.509
Protein C activity						
nETP-TMSR	0.9 [0.7, 1.2]	1.2 [0.8, 1.5]	0.001	0.9 [0.7, 1.2]	0.9 [0.6, 1.2]	0.183
nPEAK-TMSR	0.9 [0.8, 1.1]	1.1 [0.9, 1.3]	<0.001	1.0 [0.8, 1.1]	0.9 [0.8, 1.1]	0.185
nVI-TMSR	1.0 [0.9, 1.2]	1.1 [0.9, 1.2]	0.43	1.1 [0.9, 1.2]	1.0 [0.9, 1.2]	0.366

Abbreviations: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Increased TG in abacavir- vs non-abacavir-treated PLHIV

Next, we explored whether different antiretroviral drugs were associated with TG. There was no association between the use of a protease inhibitor (PI), integrase inhibitor (INSTI) or non-nucleoside reverse transcriptase inhibitor (NNRTI; figure 2) with ETP or any of the other TG parameters. In contrast, abacavir-use was associated with an increased ETP (non-abacavir: 83.7% [74.7, 97.6] vs 90.2% [82.7, 101.6], p= 0.001; figure 3 and table 2). Also, there was a trend towards higher peak thrombin concentrations in the abacavir-group (79.9% [65.2, 96.0] vs 85.8% [76.1, 95.7] p= 0.083; table 2). This effect of abacavir on ETP was independent of age, sex and inflammation (ETP: B= 8.00, p=0.006; Supplemental table 5). Tenofovir difumarate (TDF) use was the most prescribed alternative for abacavir. As a consequence, TDF was associated with a reduced ETP in our cohort (B=-7.86, p=-0.006). However, in multivariate analysis, the effect of abacavir appeared to be larger than TDF on ETP. Therefore, we attributed the effect of NRTIs on ETP to abacavir in this study. To a similar extent, we could not clearly discriminate between lamivudine and abacavir in this cohort, as this was the most prescribed combination of NRTIs (all data from multivariate analyses are shown in supplemental table 4).

As INSTI-use was high in the abacavir-group and showed a trend towards lower ETP, we corrected for INSTI use (B=-5.79, p=0.061) and the effect of abacavir on ETP became more pronounced (ETP: B=8.85, P=0.002). The abacavir-associated increase in ETP was not due to decreased protein C activity, a known inhibitor of plasmatic coagulation, as nETPsr was comparable between groups even after correction for age, sex and inflammation (abacavir: B=-0.895, p=0.37).

In most analyses on abacavir-associated cardiovascular risk in treated PLHIV, there was a discrepancy between current use, past use (>6 months) and cumulative abacavir-use (reviewed by Llibre et al [30]). Current-use of abacavir was more strongly associated to ETP in our cohort than cumulative use. When analysis was restricted to current abacavir users, cumulative exposure (in days) was not correlated with ETP (B=0.597, p=0.552).

When restricting the analysis to non-abacavir users, PLHIV had a decreased ETP, with increased lag time and a more pronounced protein C activity (table 2 and supplemental table 6) compared to uninfected controls.

In summary, our data show that abacavir-use is associated with an increase in ex vivo coagulation capacity reflected by increased TG, whereas overall, PLHIV on a non-abacavir regimen showed a decreased coagulation capacity measured by TG.

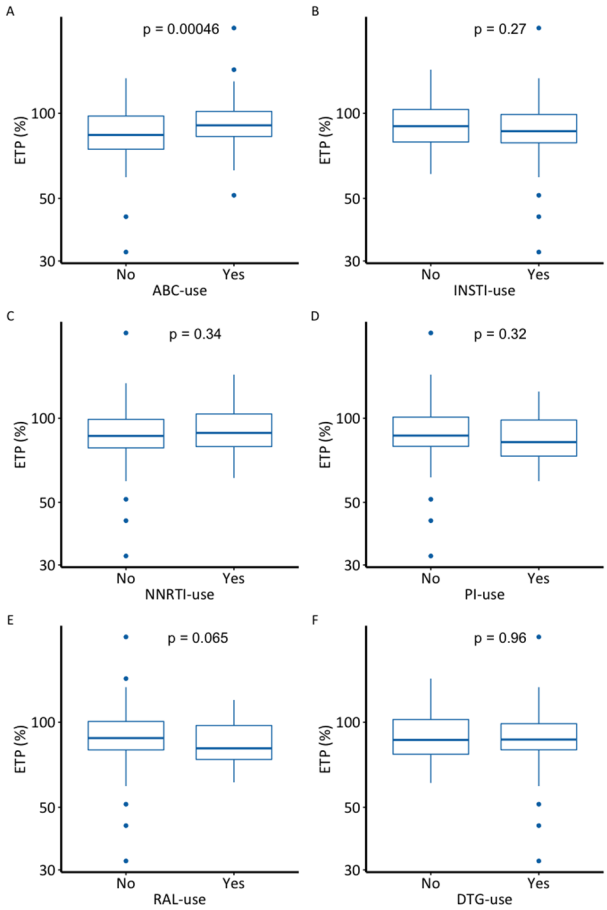

Figure 2 Endogenous thrombin potential stratified per cART-use. Endogenous thrombin potential (ETP; nM×min) were normalized as the percentage (%) of that of normal pooled plasma (NPP) tested in the same run. ABC: abacavir, INSTI: Integrase inhibitor, PI: Protease inhibitor, NNRTI: non-nucleoside reverse transcriptase inhibitor, RAL: raltegravir, DTG: Dolutegravir Values were compared using Mann-Whitney U test. Data are depicted according to Tukey. Comparisons are corrected for multiple testing using Benjamini-Hochberg procedure. ETP remained significantly higher in ABC users compared to non-ABC after FDR correction.

Mechanisms of decreased TG

Decreased TG and increased D-dimer concentrations suggest consumption of coagulation factors. Indeed, prothrombin concentrations were decreased in PLHIV (figure 3c), even after correction for age and sex (PLHIV: 109.0% [89.3, 135.1], HC: 135.1% [102.4,162.5], p<0.001; supplemental table 1). While prothrombin concentrations were decreased in PLHIV compared to controls, fibrinogen concentrations (PLHIV: 3.4mg/mL [2.8,4.0], HC: 3.2mg/mL [2.8, 4.1], p=0.746) did not differ between groups (figure 3a,c). Furthermore, prothrombin was strongly

correlated with ETP parameters in both PLHIV and uninfected controls (figure 3d and supplemental table 6) suggesting that the reduced concentration of prothrombin may underlie the observed decrease in TG in PLHIV. Reduced production of prothrombin and other coagulation factors (by the liver) was deemed unlikely due to similar concentrations of protein S (PLHIV: 91.7% [81.7, 109.4], HC: 86.9% [72.7,112.4], p=0.163, figure 3e) and fibrinogen. No difference in prothrombin or fibrinogen could be observed when PLHIV were stratified based on abacavir-use (Table 2).

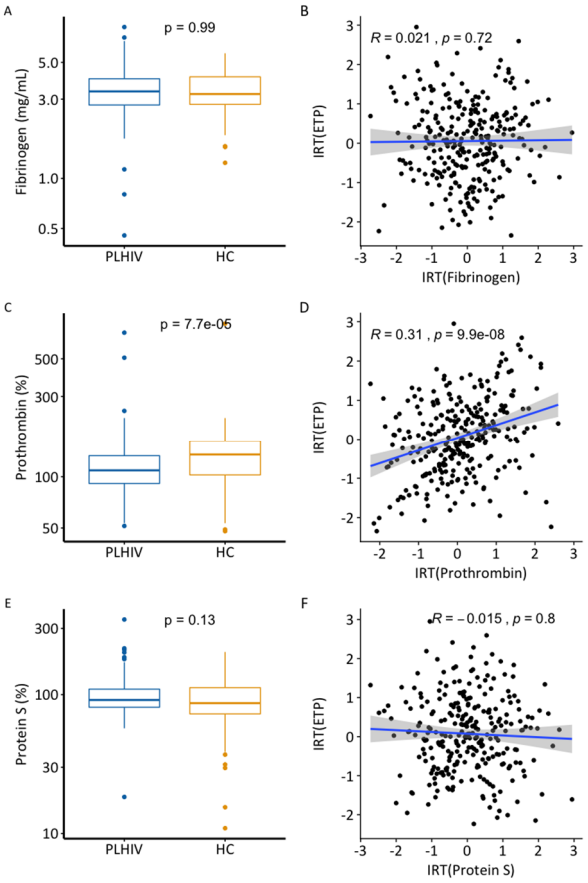

Figure 3 Coagulation factors in PLHIV and healthy controls. A) Fibrinogen concentration (mg/mL) in PLHIV and HC. B) Correlation between inverse rank-based transformed (IRT) Endogenous thrombin potential (ETP; % of normal pooled plasma (NPP)) and fibrinogen C) Prothrombin concentrations (% of NPP) in PLHIV and controls. D) Correlation plot for IRT prothrombin and IRT ETP E) Protein S concentrations (% of NPP) in PLHIV and controls. F) IRT Protein S and IRT ETP correlation plot. PLHIV: people living with HIV, HC: healthy controls. ETP: Endogenous thrombin production. Correlation coefficients are Pearson's coefficients after inverse-rank based correction (IRT) for normalization. Comparisons between cohorts were done using Mann-Whitney U-test.

DISCUSSION

In a cohort of virally suppressed PLHIV on chronic cART, we show that abacavir-use was associated with increased TG compared with non-abacavir regimens. Overall, PLHIV exhibited reduced TG compared to HIV-uninfected controls, which was associated with lower plasma concentrations of prothrombin. PLHIV had higher concentrations of inflammatory markers, which correlated with D-dimer concentrations, but not with TG parameters.

TG is a diagnostic tool for hypercoagulability states. This is not limited to venous thrombosis, and may also include arterial thrombosis[31]. The use of abacavir has been linked to an increased risk for acute cardiovascular events in different studies [10-13], but not in all [14, 15]. In contrast, abacavir use does not appear to be associated with an increased incidence of venous thrombosis[5]. Increased platelet activation was suggested to underlie the excess cardiovascular risk in users of abacavir [32-34], even though increased platelet activation was not observed in all studies [35]. A recent study found that abacavir led to an inflammatory and prothrombotic endothelial phenotype promoting *in vivo* platelet activation via endothelial microparticles (EMP) [36]. The increased TG in abacavir-use could also be caused by endothelial dysfunction and possible prothrombotic EMPs [36, 37]. In addition, thrombin directly activates platelets, so our finding of increased TG capacity in abacavir users may as such contribute to increased platelet activation and CVD [38]. In contrast to our results, a study by Jong et al [24] showed no difference in TG for abacavir-use, but only with a limited sample size of 27 individuals in the abacavir-group.

While inflammation was increased in PLHIV, we found no increase in TG. Moreover, the subgroup analysis of non-abacavir users showed a slight decrease in TG in PLHIV compared to uninfected controls. This observation is in line with earlier observations showing decreased TG in long-term treated PLHIV [25]. In this study, we confirm these results using a cohort of long-term treated PLHIV on more recently recommended cART regimens. Regarding possible mechanisms, consumption of clotting factors could play a role, as prothrombin concentrations were decreased with a concurrent increase in D-dimer in the total PLHIV group. Fibrinogen concentrations were similar across all groups, but fibrinogen is a well-known acute phase protein and could therefore reflect inflammation rather than coagulation capacity [39]. Furthermore, decreased production of (pro)coagulation factors was deemed less likely as other factors produced by the liver such as fibrinogen and protein S were similar across groups.

More recent papers have used *in silico* TG as a proxy for plasmatic coagulation potential and showed that this calculated TG correlate with development of CVD[40]. Furthermore, in the SMART study, in silico TG was lower in PLHIV without ART and ongoing viral replication compared to cART treated PLHIV [41, 42]. While D-dimer mirrors coagulation and fibrinolysis *in vivo*, *in vitro* TG reflects actual hemostatic potential of the plasmatic coagulation pathways [22]. We confirmed that D-dimers indeed correlated with endothelial activation (e.g. vWF) and inflammation (eg. sCD163), but found no such relation with TG parameters in PLHIV. This suggests that increased D-dimer concentrations are primarily influenced by increased provoking

signals for coagulation, such as endothelial activation and inflammation during HIV infection. However, increased plasmatic coagulation capacity could still play a role in cardiovascular disease in PLHIV, as was shown by a case-control study using in silico TG [42]. A significant role for plasmatic coagulation and increased TG has been shown in clinical studies including uninfected patients at risk for cardiovascular disease [31]. As PLHIV are known for increased inflammation and endothelial activation, an abacavir-associated potentiation of the plasmatic coagulation capacity could therefore still result in increased risk for arterial thrombosis.

Interestingly, protein C activity was higher in PLHIV and correlated with inflammation (sCD163) thereby reducing the overall plasmatic coagulation capacity. This rebalancing of the anti- and procoagulant pathway during HIV infection mimics the rebalancing of hemostasis seen in liver disease [43]. Monocyte and macrophage activation (sCD14 and sCD163) correlated with lag time and TTP. These parameters are mostly influenced by an increase in Tissue factor pathway inhibitor (TFPI) [44]. This TFPI is known to be increased in PLHIV [45]. Even though endothelial cells and platelets mainly produce TFPI, monocytes are known producers of TFPI too and could increase production upon activation [44].

Limitations and strengths

The cross-sectional design of our study does not allow to draw causal inferences. Furthermore, the independent increase in TG seen in abacavir treatment could be affected through indication bias whereby treatment selection is influenced by patient characteristics. Yet, in our case abacavir was preferably given to people with a decreased cardiovascular risk profile, which was reflected in our cohort with a trend towards lower CVD risk factors in the abacavir-treated group. Second, as TDF was the most prescribed alternative to abacavir, we cannot fully rule out that TDF has an inhibitory effect on TG. Yet, it will not change the conclusions that abacavir is associated with increased TG compared to non-abacavir users. To similar extent we cannot exclude the possibility that lamivudine is involved in this difference as lamivudine was prescribed concurrently to abacavir in the majority of patients. Third, we could not correlate TG parameters with clinical outcomes as was performed in the study reporting on in silico TG. While in silico TG can be calculated from plasma markers measured in (long-term) stored EDTA plasma, our functional assay of calibrated TG (CAT) requires citrated plasma, which is not regularly collected in large-scale cohorts. Actual measurement of thrombin formation using the CAT method is more precise than the modeling of the *in silico* TG method, because CAT also includes possible unknown confounders such as cART-use or other unknown factors. Therefore, CAT better reflects actual hemostatic potential in complex disease states such as an HIV infection. Furthermore, due to lower number of women in our study, generalizability of our findings to women is limited and warrants further study. Our cohort only consists of virally suppressed PLHIV on long-term treatment and reflects the current HIV-infected population. Thus, our data cannot be used to draw conclusions about patients that are still severely immune compromised or with ongoing viral replication.

In conclusion, abacavir-use was associated with increased TG and this increase could serve as an additional factor in the reported increase in thrombotic events during abacavir-use. Increased

exposure to triggers that propagate coagulation, such as inflammation, and endothelial activation likely underlie increased D-dimer concentrations found in most PLHIV**.**

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Author Contributions

WH, QdM, MR and AV designed the study. WH, LW and MJ recruited and included the participants. WH, JW, LW and performed the laboratory experiments. WH and JW analyzed the data and interpreted the data together with QdM, PG, MR and AV. WH, JW, AV and QdM wrote the manuscript. All authors have read and contributed significantly to the final manuscript.

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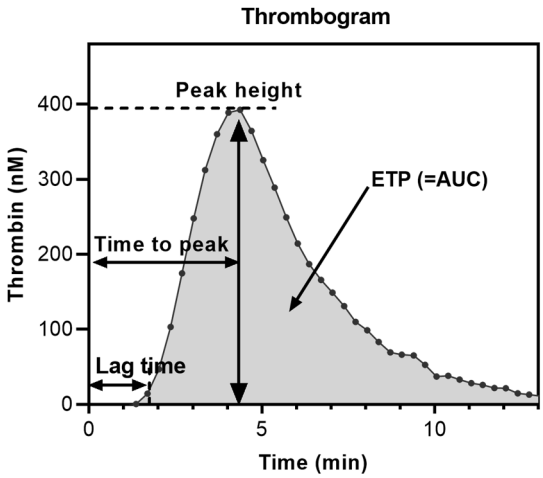
SUPPLEMENTAL



Supplemental Figure 1 The whole course of thrombin generation over time is shown as a thrombogram, which is typically characterized by 5 parameters. The time needed for thrombin to reach a detectable concentration is defined as lag time. The highest transient thrombin concentration during the reaction is referred to as peak height, and the time needed to reach this peak is called time to peak. Velocity index can be obtained by dividing peak height by the difference between time to peak and lag time. Finally, the area under the TG curve, which represents the total amount of thrombin activity during the reaction, is termed endogenous thrombin potential (ETP).

Supplemental table 1 Linear regression using age and sex as co-factors in multivariate analysis.

	Coefficient (univariate)	Coefficient (corrected for age-sex)
D-dimer	52.75 (3.73 to 101.77, p=0.035)	6.87 (-61.05 to 74.78, p=0.842)
iFABP	289.4 (123.5 to 455.3, p=0.001)	278.5 (95.3 to 461.6, p=0.003)
hsCRP	1595 (210 to 2981, p=0.024)	1863 (324 to 3402, p=0.018)
Fibrinogen	0.07 (-0.17 to 0.32, p=0.557)	-0.07 (-0.43 to 0.29, p=0.707)
vWF	13207 (5679 to 20735, p=0.001)	7120 (-3965 to 18204 p=0.207)
sCD14	423.8 (213.3 to 634.5, p<0.001)	319.9 (886.8 to 551.2, p=0.007)
sCD163	215.4 (120.1 to 310.6, p<0.001)	152.74(49.3 to 256.2, p=0.004)
Prothrombin	-21.39 (-37.29 to -5.49, p=0.009)	-23.40 (-43.15 to -3.64, p=0.020)
ProteinS	6.55 (-1.70 to 14.80, p=0.119)	-3.21 (-15.00 to 8.57, p=0.592)
LagT	0.06 (-0.06 to 0.18, p=0.336)	-0.06 (-0.23 to 0.12, p=0.525)
TTP	0.25 (0.04 to 0.47, p=0.022)	0.06 (-0.24 to 0.36, p=0.708)
Peak	-6.50 (-11.84 to -1.16, p=0.017)	-2.25 (-9.60 to 5.10, p=0.547)
VI	-19.30 (-28.72 to -9.87, p<0.001)	-7.50 (-19.93 to 4.94, p=0.236)
ETP	-6.37 (-11.06 to -1.68, p=0.008)	-4.86 (-11.39 to 1.67, p=0.144)
LagT_TM	0.02 (-0.11 to 0.14, p=0.799)	-0.07 (-0.25 to 0.11, p=0.454)
TTP_TM	0.08 (-0.06 to 0.22, p=0.270)	-0.06 (-0.26 to 0.14, p=0.569)
PEAK_TM	-12.18 (-18.97 to -5.38, p<0.001)	-3.28 (-12.34 to 5.77, p=0.476)
VI_TM	-20.94 (-31.16 to -10.72, p<0.001)	-5.43 (-18.65 to 7.79, p=0.419)
ETP_TM	-12.27 (-18.39 to -6.15, p<0.001)	-5.36 (-13.50 to 2.79, p=0.196)
nETP_TMsr	-0.20 (-0.30 to -0.09, p<0.001)	-0.08 (-0.23 to 0.06, p=0.259)
nPEAK_TMsr	-0.15 (-0.23 to -0.08, p<0.001)	-0.08 (-0.18 to 0.03, p=0.155)
nVI_TMsr	-0.01 (-0.07 to 0.06, p=0.883)	0.01 (-0.09 to 0.11, p=0.847)

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von Willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Supplemental table 2 Subgroup analysis of individuals aged 40 years and above.

	PLHIV	HC	P value
n	173	22	
AGE (mean (SD))	54.8 (7.7)	59.4 (10.3)	0.013
BMI (median [IQR])	24.4 [22.4, 26.6]	24.5 [22.3, 26.9]	0.954
d.dimer (median [IQR])	233.7 [166.2, 339.6]	333.0 [187.0, 391.9]	0.167
Plasma vWf (median [IQR])	4550 [3442, 6248]	3453 [2341, 3923]	0.003
iFABP (median [IQR])	521.0 [286.2, 748.9]	371.8 [317.0, 545.6]	0.132
hsCRP (median [IQR])	1504 [608, 3099]	812.6 [382.2, 1671.0]	0.073
sCD14 (median [IQR])	2207 [1787, 2659]	1952 [1741, 2134]	0.066
sCD163 (median [IQR])	740.9 [540.6, 904.7]	516.8 [442.7, 684.2]	0.011
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	4.0 [3.0, 4.5]	0.074
Prothrombin (median [IQR])	109.0 [88.1, 136.3]	161.5 [134.9, 180.2]	<0.001
Protein S (median [IQR])	91.7 [81.7, 111.9]	83.7 [71.3, 118.0]	0.325
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [1.7, 2.4]	0.707
TTP (median [IQR])	4.3 [4.0, 5.0]	4.7 [3.7, 5.0]	0.938
ETP (median [IQR])	86.6 [78.7, 101.0]	98.6 [94.3, 105.5]	0.014
Peak (median [IQR])	82.5 [68.8, 95.8]	90.1 [80.1, 103.4]	0.1
VI (median [IQR])	78.1 [56.0, 102.0]	90.2 [71.2, 108.8]	0.134
LagT_TM (median [IQR])	1.7 [1.4, 2.0]	2.0 [1.7, 2.0]	0.165
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	4.0 [3.6, 4.0]	0.296
ETP_TM (median [IQR])	41.1 [27.0, 51.7]	47.9 [41.0, 63.4]	0.066
PEAK_TM (median [IQR])	50.1 [31.9, 61.8]	52.4 [46.2, 68.2]	0.241
VI_TM (median [IQR])	59.6 [37.4, 82.3]	65.2 [55.6, 83.8]	0.207
nETP_TMsr (median [IQR])	0.9 [0.7, 1.2]	0.9 [0.7, 1.3]	0.558
nPEAK_TMsr (median [IQR])	0.9 [0.8, 1.1]	0.9 [0.8, 1.2]	0.494
nVI_TMsr (median [IQR])	1.0 [0.9, 1.3]	1.0 [0.9, 1.3]	0.97

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Supplemental table 3; Subgroup analysis restricting to males only.

	PLHIV	HC	P value
n	190	34	
AGE (mean (SD))	51.2 (11.0)	39.7 (17.7)	<0.001
BMI (median [IQR])	24.1 [22.0, 25.9]	23.9 [21.9, 25.4]	0.89
d.dimerl (median [IQR])	216.5 [158.0, 334.2]	166.9 [115.2, 275.4]	0.019
Plasma vWf (median [IQR])	42675 [31896, 58881]	31559 [26013, 45904]	0.011
iFABP (median [IQR])	478.1 [245.5, 694.7]	268.3 [134.6, 397.5]	0.003
hsCRP (median [IQR])	1451 [607, 2688]	675 [166, 1309]	0.003
sCD14 (median [IQR])	2139 [1779, 2630]	1851 [1614, 2172]	0.007
sCD163 (median [IQR])	700.2 [516.8, 900.0]	532.1 [418.3, 620.5]	0.002
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	3.3 [2.8, 4.0]	0.615
Prothrombin (median [IQR])	109.1 [89.3, 136.7]	136.7 [110.6, 162.1]	0.003
ProteinS (median [IQR])	91.7 [80.3, 111.3]	99.7 [79.9, 133.6]	0.315
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [2.0, 2.3]	0.052
TTP (median [IQR])	4.4 [4.0, 5.0]	4.7 [4.0, 5.0]	0.329
ETP (median [IQR])	86.6 [78.6, 99.2]	86.8 [80.5, 98.7]	0.961
Peak (median [IQR])	82.4 [68.7, 95.4]	78.4 [66.3, 96.8]	0.588
VI (median [IQR])	76.8 [55.9, 100.7]	76.1 [56.2, 104.6]	0.813
LagT_TM (median [IQR])	1.7 [1.4, 2.0]	2.0 [1.7, 2.0]	0.029
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	4.0 [3.7, 4.0]	0.082
ETP_TM (median [IQR])	40.6 [27.7, 52.0]	43.5 [30.0, 50.5]	0.764
PEAK_TM (median [IQR])	49.5 [31.8, 62.2]	49.9 [37.9, 62.2]	0.898
VI_TM (median [IQR])	59.6 [38.2, 80.2]	60.2 [44.2, 81.7]	0.691
nETP_TMsr (median [IQR])	0.9 [0.7, 1.2]	0.9 [0.7, 1.3]	0.594
nPEAK_TMsr (median [IQR])	0.9 [0.8, 1.1]	1.0 [0.8, 1.2]	0.347
nVI_TMsr (median [IQR])	1.1 [0.9, 1.2]	1.1 [0.9, 1.2]	0.711

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Supplemental table 4; Univariate and multivariate analysis of circulating markers.

	Univariate	Model 1 (Age-sex)	model (age-sex-sCD14)
ETP			
Prothrombin	4.45 (0.79 to 8.11, p=0.018)	5.81 (1.92 to 9.70, p=0.004)	5.90 (2.02 to 9.79, p=0.003)
Fibrinogen	-6.28 (-9.73 to -2.82, p<0.001)	-6.56 (-10.26 to -2.87, p=0.001)	-6.41 (-10.13 to -2.69, p=0.001)
sCD163	1.96 (-4.54 to 8.46, p=0.553)	-2.56 (-10.29 to 5.17, p=0.513)	-1.54 (-9.41 to 6.33, p=0.700)
sCD14	-1.90 (-11.49 to 7.69, p=0.697)	-8.07 (-19.47 to 3.33, p=0.164)	-8.07 (-19.47 to 3.33, p=0.164)
hsCRP	1.48 (-0.61 to 3.58, p=0.165)	0.43 (-1.99 to 2.84, p=0.727)	0.77 (-1.72 to 3.25, p=0.543)
iFABP	-2.35 (-5.13 to 0.43, p=0.097)	-2.87 (-5.98 to 0.24, p=0.071)	-2.84 (-5.94 to 0.26, p=0.072)
Peak			
Prothrombin	1.03 (-3.32 to 5.39, p=0.640)	2.16 (-2.58 to 6.90, p=0.370)	2.28 (-2.47 to 7.02, p=0.345)
Fibrinogen	-5.23 (-9.39 to -1.08, p=0.014)	-6.15 (-10.65 to -1.65, p=0.008)	-5.96 (-10.50 to -1.43, p=0.010)
sCD163	-4.71 (-12.30 to 2.87, p=0.222)	-8.11 (-17.20 to 0.98, p=0.080)	-7.18 (-16.45 to 2.09, p=0.128)
sCD14	-6.34 (-17.53 to 4.86, p=0.266)	-9.07 (-22.58 to 4.45, p=0.187)	-9.07 (-22.58 to 4.45, p=0.187)
hsCRP	0.48 (-2.00 to 2.97, p=0.701)	-0.24 (-3.13 to 2.65, p=0.870)	0.18 (-2.79 to 3.15, p=0.906)
iFABP	-2.99 (-6.23 to 0.26, p=0.071)	-2.99 (-6.69 to 0.70, p=0.112)	-2.97 (-6.65 to 0.72, p=0.114)
TTP			
Prothrombin	0.10 (-0.08 to 0.29, p=0.283)	0.08 (-0.12 to 0.29, p=0.409)	0.08 (-0.12 to 0.27, p=0.457)
Fibrinogen	0.15 (-0.02 to 0.33, p=0.089)	0.23 (0.04 to 0.41, p=0.018)	0.21 (0.02 to 0.40, p=0.027)
sCD163	0.51 (0.20 to 0.82, p=0.001)	0.49 (0.12 to 0.86, p=0.010)	0.42 (0.04 to 0.79, p=0.029)
sCD14	0.69 (0.23 to 1.15, p=0.003)	0.63 (0.08 to 1.19, p=0.024)	0.63 (0.08 to 1.19, p=0.024)
hsCRP	0.12 (0.02 to 0.22, p=0.023)	0.09 (-0.02 to 0.21, p=0.114)	0.07 (-0.05 to 0.19, p=0.264)
iFABP	0.07 (-0.07 to 0.20, p=0.336)	0.06 (-0.09 to 0.22, p=0.420)	0.06 (-0.09 to 0.21, p=0.429)
LagTime			
Prothrombin	-0.00 (-0.11 to 0.10, p=0.933)	-0.03 (-0.15 to 0.09, p=0.611)	-0.04 (-0.15 to 0.07, p=0.501)
Fibrinogen	0.15 (0.05 to 0.25, p=0.004)	0.20 (0.09 to 0.30, p<0.001)	0.18 (0.08 to 0.28, p=0.001)
sCD163	0.25 (0.07 to 0.43, p=0.007)	0.17 (-0.04 to 0.39, p=0.119)	0.09 (-0.12 to 0.30, p=0.395)
sCD14	0.64 (0.38 to 0.90, p<0.001)	0.62 (0.32 to 0.93, p<0.001)	0.62 (0.32 to 0.93, p<0.001)
hsCRP	0.10 (0.05 to 0.16, p=0.001)	0.08 (0.02 to 0.15, p=0.015)	0.06 (-0.01 to 0.12, p=0.087)
iFABP	0.01 (-0.07 to 0.09, p=0.809)	0.02 (-0.07 to 0.11, p=0.704)	0.02 (-0.07 to 0.10, p=0.724)
nETP-TMsr			
Prothrombin	-0.07 (-0.16 to 0.01, p=0.105)	-0.07 (-0.16 to 0.01, p=0.096)	-0.07 (-0.16 to 0.01, p=0.102)
Fibrinogen	-0.06 (-0.16 to 0.03, p=0.188)	-0.08 (-0.18 to 0.02, p=0.113)	-0.08 (-0.18 to 0.02, p=0.111)
sCD163	-0.21 (-0.37 to -0.04, p=0.014)	-0.20 (-0.38 to -0.02, p=0.032)	-0.20 (-0.39 to -0.02, p=0.032)
sCD14	-0.19 (-0.43 to 0.05, p=0.118)	-0.02 (-0.30 to 0.26, p=0.885)	-0.02 (-0.30 to 0.26, p=0.885)
hsCRP	-0.07 (-0.12 to -0.02, p=0.009)	-0.06 (-0.11 to -0.00, p=0.033)	-0.06 (-0.11 to -0.00, p=0.038)
iFABP	-0.01 (-0.08 to 0.06, p=0.817)	-0.01 (-0.08 to 0.06, p=0.802)	-0.01 (-0.08 to 0.06, p=0.812)
IRT_ETP			
Prothrombin	0.22 (0.03 to 0.41, p=0.026)	0.30 (0.10 to 0.49, p=0.003)	0.27 (0.08 to 0.46, p=0.006)
Fibrinogen	-0.30 (-0.48 to -0.12, p=0.001)	-0.31 (-0.49 to -0.12, p=0.001)	-0.28 (-0.46 to -0.10, p=0.003)

sCD163	0.19 (-0.15 to 0.52, p=0.268)	-0.06 (-0.45 to 0.32, p=0.754)	0.13 (-0.26 to 0.52, p=0.509)
sCD14	-0.07 (-0.57 to 0.42, p=0.779)	-0.47 (-1.04 to 0.10, p=0.104)	0.42 (0.14 to 0.70, p=0.004)
hsCRP	0.11 (0.00 to 0.22, p=0.049)	0.04 (-0.08 to 0.16, p=0.466)	0.08 (-0.04 to 0.20, p=0.201)
iFABP	-0.12 (-0.26 to 0.03, p=0.109)	-0.15 (-0.30 to 0.01, p=0.066)	-0.12 (-0.27 to 0.04, p=0.133)

Linear regression was used for analysis. Data shown as Beta with confidence interval and p-value. Model 1 includes age ,time of inclusion and sex as covariates. Model 2 also includes sCD14 as a covariate. High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP.

Supplemental table 5; Univariate and multivariate analysis of thrombin generation parameters (by calibrated thrombin generation).

	Univariate	model 1 (age-sex)	model 2 (age-sex-sCD14)	model 3 (age-sex-sCD14-ABC)
ETP				
Smoking (pckyrs)	-0.07 (-0.19 to 0.05, p=0.255)	-0.11 (-0.25 to 0.04, p=0.148)	-0.10 (-0.25 to 0.05, p=0.178)	-0.11 (-0.25 to 0.04, p=0.148)
HIV duration	0.46 (-0.24 to 1.16, p=0.198)	0.52 (-0.28 to 1.32, p=0.203)	0.50 (-0.31 to 1.30, p=0.226)	0.52 (-0.28 to 1.32, p=0.203)
cART duration	0.52 (-0.40 to 1.44, p=0.264)	0.43 (-0.60 to 1.47, p=0.409)	0.47 (-0.57 to 1.50, p=0.376)	0.43 (-0.60 to 1.47, p=0.409)
HIV RNA zenith	-0.09 (-0.29 to 0.12, p=0.414)	-0.15 (-0.43 to 0.12, p=0.271)	-0.15 (-0.42 to 0.13, p=0.291)	-0.15 (-0.43 to 0.12, p=0.271)
CD4 nadir	1.44 (-1.74 to 4.61, p=0.374)	2.90 (-0.83 to 6.63, p=0.127)	2.86 (-0.94 to 6.65, p=0.139)	2.90 (-0.83 to 6.63, p=0.127)
CD4 count	1.56 (-0.99 to 4.12, p=0.229)	1.55 (-1.44 to 4.53, p=0.307)	1.36 (-1.65 to 4.37, p=0.373)	1.55 (-1.44 to 4.53, p=0.307)
CD4-CD8 ratio	0.47 (-0.99 to 1.94, p=0.522)	0.09 (-1.53 to 1.70, p=0.914)	0.09 (-1.52 to 1.71, p=0.908)	0.09 (-1.53 to 1.70, p=0.914)
CD4 recovery (relative)	7.85 (1.41 to 14.29, p=0.017)	9.30 (2.05 to 16.55, p=0.012)	8.68 (1.33 to 16.03, p=0.021)	9.30 (2.05 to 16.55, p=0.012)
INSTI	-2.99 (-8.36 to 2.38, p=0.273)	-3.63 (-9.77 to 2.52, p=0.245)	-4.18 (-10.35 to 1.99, p=0.182)	-3.63 (-9.77 to 2.52, p=0.245)
Raltegravir	-4.95 (-11.49 to 1.59, p=0.137)	-4.98 (-12.79 to 2.82, p=0.209)	-5.75 (-13.60 to 2.10, p=0.150)	-4.98 (-12.79 to 2.82, p=0.209)
Elvitegravir	3.72 (-5.87 to 13.30, p=0.445)	4.19 (-6.49 to 14.87, p=0.440)	3.87 (-6.84 to 14.57, p=0.477)	4.19 (-6.49 to 14.87, p=0.440)
Dolutegravir	-0.42 (-5.52 to 4.68, p=0.871)	-1.25 (-7.06 to 4.57, p=0.673)	-1.19 (-7.03 to 4.64, p=0.687)	-1.25 (-7.06 to 4.57, p=0.673)
PI	-3.01 (-9.98 to 3.96, p=0.395)	-2.70 (-11.03 to 5.64, p=0.524)	-2.78 (-11.11 to 5.56, p=0.512)	-2.70 (-11.03 to 5.64, p=0.524)
NNRTI	2.96 (-2.58 to 8.51, p=0.293)	4.57 (-1.72 to 10.86, p=0.153)	5.27 (-1.06 to 11.60, p=0.102)	4.57 (-1.72 to 10.86, p=0.153)
Tenofovir (TDF)	-8.01 (-12.95 to -3.07, p=0.002)	-7.64 (-13.24 to -2.04, p=0.008)	-7.86 (-13.48 to -2.25, p=0.006)	-7.64 (-13.24 to -2.04, p=0.008)
Lamivudine	8.69 (3.81 to 13.58, p=0.001)	8.53 (3.04 to 14.01, p=0.003)	8.51 (3.02 to 14.01, p=0.003)	8.53 (3.04 to 14.01, p=0.003)
Emtricitabine	-8.41 (-13.35 to -3.47, p=0.001)	-8.67 (-14.24 to -3.11, p=0.002)	-8.98 (-14.55 to -3.41, p=0.002)	-8.67 (-14.24 to -3.11, p=0.002)
Abacavir	8.40 (3.48 to 13.32, p=0.001)	7.68 (2.07 to 13.29, p=0.008)	8.00 (2.38 to 13.63, p=0.006)	NA
Peak				
Smoking (pckyrs)	-0.12 (-0.26 to 0.02, p=0.090)	-0.14 (-0.31 to 0.03, p=0.115)	-0.13 (-0.30 to 0.04, p=0.143)	-0.12 (-0.30 to 0.05, p=0.160)
HIV duration	0.20 (-0.62 to 1.02, p=0.634)	0.25 (-0.70 to 1.20, p=0.608)	0.21 (-0.75 to 1.17, p=0.667)	0.19 (-0.76 to 1.15, p=0.690)

cART duration	0.51 (-0.57 to 1.58, p=0.352)	0.31 (-0.91 to 1.54, p=0.616)	0.34 (-0.89 to 1.57, p=0.582)	0.43 (-0.80 to 1.67, p=0.490)
HIV RNA zenith	-0.20 (-0.45 to 0.04, p=0.098)	-0.30 (-0.62 to 0.02, p=0.066)	-0.30 (-0.62 to 0.03, p=0.071)	-0.29 (-0.61 to 0.03, p=0.079)
CD4 nadir	1.27 (-2.45 to 4.99, p=0.502)	2.24 (-2.19 to 6.68, p=0.320)	2.03 (-2.49 to 6.55, p=0.377)	2.56 (-2.03 to 7.15, p=0.273)
CD4 count	3.27 (0.30 to 6.23, p=0.031)	3.54 (0.03 to 7.04, p=0.048)	3.43 (-0.11 to 6.96, p=0.057)	3.34 (-0.20 to 6.89, p=0.064)
CD4-CD8 ratio	0.48 (-1.24 to 2.20, p=0.581)	0.22 (-1.70 to 2.14, p=0.821)	0.22 (-1.71 to 2.15, p=0.821)	0.25 (-1.69 to 2.18, p=0.802)
CD4 recovery (relative)	9.78 (2.26 to 17.30, p=0.011)	11.32 (2.75 to 19.90, p=0.010)	10.75 (2.05 to 19.45, p=0.016)	10.69 (1.99 to 19.39, p=0.016)
INSTI	-6.45 (-12.69 to -0.21, p=0.043)	-7.08 (-14.30 to 0.14, p=0.054)	-7.70 (-14.95 to -0.44, p=0.038)	-8.57 (-15.91 to -1.24, p=0.022)
Raltegravir	-5.35 (-13.01 to 2.30, p=0.170)	-6.36 (-15.59 to 2.88, p=0.176)	-7.25 (-16.55 to 2.05, p=0.126)	-6.66 (-16.08 to 2.75, p=0.164)
Elvitegravir	6.83 (-4.36 to 18.02, p=0.230)	8.03 (-4.58 to 20.64, p=0.210)	7.60 (-5.06 to 20.26, p=0.237)	10.58 (-2.64 to 23.81, p=0.116)
Dolutegravir	-3.85 (-9.79 to 2.09, p=0.202)	-4.44 (-11.30 to 2.42, p=0.203)	-4.32 (-11.20 to 2.57, p=0.218)	-7.35 (-14.97 to 0.27, p=0.059)
PI	0.57 (-7.59 to 8.74, p=0.890)	0.06 (-9.82 to 9.95, p=0.990)	-0.06 (-9.96 to 9.84, p=0.990)	0.86 (-9.18 to 10.91, p=0.865)
NNRTI	5.61 (-0.85 to 12.07, p=0.088)	8.17 (0.78 to 15.56, p=0.030)	8.97 (1.53 to 16.40, p=0.018)	10.26 (2.69 to 17.83, p=0.008)
Tenofovir (TDF)	-4.88 (-10.76 to 1.01, p=0.104)	-4.57 (-11.31 to 2.18, p=0.183)	-4.86 (-11.63 to 1.91, p=0.158)	-5.29 (-16.19 to 5.61, p=0.340)
Lamivudine	5.46 (-0.38 to 11.29, p=0.067)	5.12 (-1.51 to 11.76, p=0.129)	5.17 (-1.48 to 11.82, p=0.127)	7.32 (-4.80 to 19.45, p=0.235)
Emtricitabine	-5.95 (-11.83 to -0.07, p=0.047)	-6.25 (-12.96 to 0.45, p=0.067)	-6.64 (-13.37 to 0.09, p=0.053)	-10.60 (-21.94 to 0.73, p=0.067)
Abacavir	4.44 (-1.44 to 10.32, p=0.138)	3.22 (-3.55 to 9.99, p=0.349)	3.60 (-3.20 to 10.41, p=0.297)	NA
LagTime				
Smoking (pckyrs)	0.00 (-0.00 to 0.01, p=0.055)	0.00 (-0.00 to 0.01, p=0.052)	0.00 (-0.00 to 0.01, p=0.104)	0.00 (-0.00 to 0.01, p=0.100)
HIV duration	0.01 (-0.01 to 0.03, p=0.274)	0.00 (-0.02 to 0.03, p=0.739)	0.01 (-0.01 to 0.03, p=0.533)	0.01 (-0.02 to 0.03, p=0.539)
cART duration	-0.01 (-0.04 to 0.01, p=0.309)	-0.01 (-0.04 to 0.02, p=0.354)	-0.02 (-0.04 to 0.01, p=0.270)	-0.02 (-0.04 to 0.01, p=0.281)
HIV RNA zenith	0.00 (-0.00 to 0.01, p=0.135)	0.01 (-0.00 to 0.01, p=0.109)	0.01 (-0.00 to 0.01, p=0.115)	0.01 (-0.00 to 0.01, p=0.113)
CD4 nadir	0.02 (-0.07 to 0.11, p=0.611)	-0.00 (-0.11 to 0.10, p=0.951)	0.02 (-0.08 to 0.12, p=0.702)	0.02 (-0.08 to 0.13, p=0.665)
CD4 count	-0.07 (-0.14 to 0.00, p=0.065)	-0.07 (-0.16 to 0.01, p=0.078)	-0.07 (-0.15 to 0.01, p=0.106)	-0.07 (-0.15 to 0.01, p=0.104)
CD4-CD8 ratio	-0.05 (-0.09 to -0.01, p=0.015)	-0.06 (-0.10 to -0.02, p=0.007)	-0.06 (-0.10 to -0.02, p=0.006)	-0.06 (-0.10 to -0.02, p=0.006)
CD4 recovery (relative)	-0.24 (-0.43 to -0.06, p=0.009)	-0.30 (-0.50 to -0.10, p=0.004)	-0.25 (-0.45 to -0.05, p=0.013)	-0.25 (-0.45 to -0.05, p=0.014)
INSTI	0.08 (-0.08 to 0.23, p=0.319)	0.15 (-0.02 to 0.33, p=0.079)	0.19 (0.03 to 0.36, p=0.024)	0.19 (0.03 to 0.36, p=0.025)
Raltegravir	0.13 (-0.06 to 0.32, p=0.175)	0.22 (0.01 to 0.44, p=0.044)	0.28 (0.07 to 0.49, p=0.008)	0.29 (0.08 to 0.51, p=0.007)
Elvitegravir	-0.38 (-0.65 to -0.11, p=0.006)	-0.34 (-0.64 to -0.05, p=0.023)	-0.31 (-0.60 to -0.03, p=0.033)	-0.33 (-0.63 to -0.03, p=0.031)
Dolutegravir	0.09 (-0.05 to 0.24, p=0.213)	0.11 (-0.05 to 0.27, p=0.187)	0.10 (-0.06 to 0.26, p=0.220)	0.11 (-0.06 to 0.29, p=0.213)
PI	0.21 (0.02 to 0.41, p=0.033)	0.19 (-0.04 to 0.42, p=0.111)	0.20 (-0.02 to 0.42, p=0.080)	0.21 (-0.02 to 0.44, p=0.070)
NNRTI	-0.13 (-0.29 to 0.02, p=0.095)	-0.19 (-0.36 to -0.01, p=0.038)	-0.23 (-0.40 to -0.07, p=0.007)	-0.24 (-0.41 to -0.07, p=0.007)

Tenofovir (TDF)	-0.04 (-0.19 to 0.10, p=0.568)	-0.02 (-0.19 to 0.14, p=0.764)	-0.00 (-0.16 to 0.15, p=0.950)	0.03 (-0.22 to 0.28, p=0.840)
Lamivudine	0.02 (-0.12 to 0.16, p=0.782)	0.01 (-0.15 to 0.17, p=0.890)	0.01 (-0.15 to 0.16, p=0.922)	-0.03 (-0.31 to 0.25, p=0.853)
Emtricitabine	-0.08 (-0.22 to 0.07, p=0.283)	-0.07 (-0.23 to 0.09, p=0.418)	-0.04 (-0.20 to 0.11, p=0.591)	-0.08 (-0.34 to 0.19, p=0.563)
Abacavir	0.03 (-0.11 to 0.18, p=0.668)	0.04 (-0.12 to 0.21, p=0.586)	0.02 (-0.14 to 0.17, p=0.810)	NA
nETP-TMsr				
Smoking (pckyrs)	-0.00 (-0.01 to -0.00, p=0.013)	-0.00 (-0.01 to -0.00, p=0.019)	-0.00 (-0.01 to -0.00, p=0.021)	-0.00 (-0.01 to -0.00, p=0.018)
HIV duration	-0.02 (-0.04 to -0.00, p=0.028)	-0.02 (-0.04 to 0.00, p=0.063)	-0.02 (-0.04 to 0.00, p=0.055)	-0.02 (-0.04 to 0.00, p=0.064)
cART duration	0.01 (-0.01 to 0.04, p=0.318)	0.01 (-0.02 to 0.04, p=0.501)	0.01 (-0.02 to 0.04, p=0.518)	0.01 (-0.02 to 0.04, p=0.561)
HIV RNA zenith	-0.01 (-0.01 to -0.00, p=0.006)	-0.01 (-0.01 to -0.00, p=0.017)	-0.01 (-0.01 to -0.00, p=0.017)	-0.01 (-0.01 to -0.00, p=0.016)
CD4 nadir	-0.01 (-0.08 to 0.07, p=0.887)	-0.00 (-0.09 to 0.08, p=0.915)	-0.01 (-0.09 to 0.08, p=0.823)	-0.02 (-0.10 to 0.07, p=0.691)
CD4 count	0.10 (0.04 to 0.16, p=0.002)	0.10 (0.03 to 0.16, p=0.005)	0.10 (0.03 to 0.17, p=0.004)	0.10 (0.03 to 0.17, p=0.003)
CD4-CD8 ratio	-0.02 (-0.05 to 0.02, p=0.355)	-0.01 (-0.05 to 0.03, p=0.633)	-0.01 (-0.05 to 0.03, p=0.639)	-0.01 (-0.05 to 0.03, p=0.594)
CD4 recovery (relative)	0.10 (-0.07 to 0.27, p=0.236)	0.09 (-0.09 to 0.26, p=0.321)	0.09 (-0.09 to 0.27, p=0.300)	0.09 (-0.09 to 0.27, p=0.315)
INSTI	-0.08 (-0.22 to 0.05, p=0.234)	-0.12 (-0.26 to 0.02, p=0.102)	-0.12 (-0.26 to 0.03, p=0.106)	-0.11 (-0.25 to 0.03, p=0.131)
Raltegravir	-0.10 (-0.27 to 0.08, p=0.267)	-0.17 (-0.35 to 0.01, p=0.061)	-0.17 (-0.36 to 0.01, p=0.060)	-0.19 (-0.37 to -0.00, p=0.045)
Elvitegravir	0.22 (-0.00 to 0.45, p=0.054)	0.19 (-0.04 to 0.42, p=0.097)	0.19 (-0.04 to 0.42, p=0.104)	0.18 (-0.07 to 0.42, p=0.153)
Dolutegravir	-0.08 (-0.20 to 0.05, p=0.234)	-0.07 (-0.20 to 0.07, p=0.331)	-0.06 (-0.20 to 0.07, p=0.354)	-0.05 (-0.20 to 0.10, p=0.531)
PI	-0.09 (-0.26 to 0.09, p=0.321)	-0.10 (-0.28 to 0.08, p=0.271)	-0.10 (-0.29 to 0.08, p=0.268)	-0.12 (-0.31 to 0.07, p=0.205)
NNRTI	0.09 (-0.05 to 0.23, p=0.193)	0.13 (-0.01 to 0.28, p=0.069)	0.14 (-0.01 to 0.28, p=0.070)	0.13 (-0.02 to 0.28, p=0.093)
Tenofovir (TDF)	0.08 (-0.05 to 0.20, p=0.233)	0.06 (-0.07 to 0.19, p=0.336)	0.06 (-0.07 to 0.19, p=0.359)	0.04 (-0.16 to 0.25, p=0.667)
Lamivudine	-0.05 (-0.18 to 0.08, p=0.424)	-0.05 (-0.18 to 0.08, p=0.478)	-0.04 (-0.17 to 0.09, p=0.509)	0.01 (-0.22 to 0.24, p=0.959)
Emtricitabine	0.04 (-0.08 to 0.17, p=0.501)	0.03 (-0.10 to 0.16, p=0.691)	0.02 (-0.11 to 0.16, p=0.726)	-0.05 (-0.27 to 0.16, p=0.618)
Abacavir	-0.07 (-0.19 to 0.06, p=0.307)	-0.06 (-0.19 to 0.07, p=0.372)	-0.06 (-0.19 to 0.08, p=0.402)	NA

Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (nETPTMsr).Linear regression was used for analysis. Data shown as Beta with confidence interval and p-value. Model 1 includes age, time of inclusion and sex as covariates. Model 2 also includes sCD14 as a covariate. Model 3 includes abacavir use as covariate.

Supplemental table 6. Subgroup analysis restricting to PLHIV on a non-abacavir (ABC) containing regimen and healthy controls only

	PLHIV-no ABC	HC	P value
n	114	56	
AGE (mean (SD))	52.3 (10.3)	39.9 (17.3)	<0.001
BMI (median [IQR])	24.2 [22.4, 26.0]	23.8 [21.5, 25.6]	0.395
Sex (%female)	13 (11.4)	22 (39.3)	<0.001
iFABP (median [IQR])	576.2 [287.4, 897.2]	242.5 [112.9, 376.9]	<0.001
hsCRP (median [IQR])	1558 [624, 3285]	651.2 [205.9, 1179.2]	<0.001
sCD14 (median [IQR])	2063 [1745, 2591]	1789 [1502, 2071]	0.001
sCD163 (median [IQR])	765.8 [581.6, 916.1]	517.3 [410.7, 578.1]	<0.001
Plasma vWf (median [IQR])	42344.2 [31562.4, 61642.5]	32468.9 [2441.6, 43935.1]	<0.001
d.dimer (median [IQR])	213.4 [158.6, 335.5]	170.5 [122.0, 307.2]	0.01
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	3.2 [2.8, 4.1]	0.589
Prothrombin (median [IQR])	106.0 [87.1, 135.1]	135.1 [102.4, 162.5]	<0.001
ProteinS (median [IQR])	91.7 [81.7, 109.4]	86.9 [72.7, 112.4]	0.163
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [1.7, 2.0]	0.618
TTP (median [IQR])	4.3 [4.0, 5.0]	4.3 [3.5, 5.0]	0.103
ETP (median [IQR])	83.7 [74.7, 97.6]	93.0 [83.3, 105.0]	<0.001
Peak (median [IQR])	79.9 [65.2, 96.0]	88.1 [72.3, 101.5]	0.012
VI (median [IQR])	76.4 [53.6, 102.5]	97.3 [64.0, 123.5]	0.002
LagT_TM (median [IQR])	1.7 [1.4, 2.3]	1.7 [1.4, 2.0]	0.996
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.687
ETP_TM (median [IQR])	41.0 [28.0, 51.1]	50.1 [32.8, 76.2]	0.003
PEAK_TM (median [IQR])	50.9 [33.2, 61.6]	57.1 [39.8, 82.5]	0.011
VI_TM (median [IQR])	62.1 [39.8, 85.4]	71.9 [51.8, 112.5]	0.004
nETP_TMsr (median [IQR])	0.9 [0.7, 1.2]	1.2 [0.8, 1.5]	0.012
nPEAK_TMsr (median [IQR])	1.0 [0.8, 1.1]	1.1 [0.9, 1.3]	0.003

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

CHAPTER 7

Kallikrein augments the anticoagulant function of the protein C system in thrombin generation

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ABSTRACT

Background & Aims

Genetics play a significant role in coagulation phenotype and venous thromboembolism risk. Resistance to the anticoagulant activated protein C (APC) is an established risk for thrombosis. We explored the genetic determinants of thrombin generation (TG) and thrombomodulin (TM)-modulated TG.

Methods

Platelet poor plasma of healthy individuals of Western-European background from the Human Functional Genomics Project was used. Tissue factor-triggered TG was measured with the Calibrated Automated Thrombography (CAT) assay. TM was supplemented into a parallel TG measurement to assess the protein C pathway function. Genetic determinants of TG parameters and protein C pathway function were assessed using genome-wide single-nucleotide polymorphism (SNP) genotyping. Functional assays were performed to verify the effect of prekallikrein, kallikrein and apolipoprotein A-IV on TM- and APC- modified TG.

Results

TG data from 392 individuals were analysed, and genotyping showed that the *KLKB1* gene (top SNP ID: rs4241819) on chromosome 4 was significantly associated at genome-wide level with the inhibitory capacity of TM on endogenous thrombin potential (nETP_TMsr, $p = 4.27 \times 10^{-8}$). In vitro supplementation of kallikrein, but not prekallikrein or apolipoprotein A-IV, into plasma dose-dependently augmented the anticoagulant effect of TM and APC in TG: 120 nM kallikrein resulted in approximately 30% stronger inhibition of thrombin formation by TM or APC.

Conclusions

Our results suggest that kallikrein plays a role in the regulation of the anticoagulant protein C pathway in TG, which may provide a possible mechanism for the previously observed association between the *KLKB1* gene and venous thrombosis.

INTRODUCTION

The coagulation system, a complex machinery consisting of a serial of pro- and anti-coagulant (pro)enzymes and cofactors, is responsible for the prevention of excessive blood loss and the formation of unnecessary thrombi. Kallikrein (PKa) is a component of the contact activation system, together with FXII and high molecular weight kininogen. Prekallikrein (PK) is the precursor of the trypsin-like plasma protease PKa and circulates in plasma in complex with HK at a concentration of approximately 580 nM. PK is activated to PKa by FXIIa, which then results in the reciprocal activation of both enzymes and subsequently leads to the activation of FXI and downstream thrombin generation (TG) [1].

The protein C system is an important anticoagulant pathway [2]. During coagulation, thrombin binds to the endothelial receptor thrombomodulin (TM) and activates protein C to its active form (APC). APC proteolytically inactivates factor (F) Va and FVIIIa and reduces further thrombin formation [2, 3]. Dysfunction of the protein C pathway is a common cause of thrombophilia[4].

The capacity of a plasma to form thrombin, as reflected by the endogenous thrombin potential (ETP) in calibrated thrombin generation tests [5], is a crucial determinant of blood coagulability and was shown to be predictive of bleeding or thrombosis [6-8]. Modification of TG assays with exogenous APC or TM makes them sensitive to dysfunctions of the protein C pathway, including protein S/C deficiency, FV_{Leiden} and prothrombin G20210A [9].

Previous studies demonstrated that TG profiles could serve as an intermediate phenotype to discover genetic variants related with venous thromboembolism (VTE) [10-13]. Segers et al showed that several established thrombophilic mutations including FV_{Leiden} and prothrombin G20210A were related with enhanced TG or APC-modified TG in a group of healthy donors [12], which was also confirmed in 188 FV_{Leiden} heterozygotes carriers [11]. Importantly, using this approach, Rocanin-Arjo et al discovered in the MARTHA study and two replication studies that the *ORM1* gene is a locus associated with the lag time of TG, and further functional studies confirmed that higher orosomucoid concentrations impaired TG [13].

In this study, we sought to use TM-modified TG profiles to discover novel genetic determinants of TG and the protein C pathway function.

MATERIALS AND METHODS

Study population

The study population is part of the previously described 500 Human Functional Genomics Project (500FG)[14, 15]. This study was approved by the local Ethical Committee (NL42561.091.12, 2012/550) and was conducted according to the Declaration of Helsinki and in accordance with the Dutch Medical Research involving Human Subjects Act. Between August 2013 and

December 2014 healthy adult individuals of Western-European origin were recruited at Radboud University Medical Centre, Nijmegen, the Netherlands. Exclusion criteria were pregnancy, breastfeeding, the use of any medication in the past month, and acute or chronic diseases. All participants gave written informed consent before blood was drawn into 3.2% sodium citrate Vacutainer tubes (Becton Dickinson). Platelet poor plasma (PPP) was prepared by centrifugation (2000g for 10 minutes) within 2 hours post blood draw and stored at −80°C.

PPP-TG measurement

A modified Calibrated Automated Thrombography (MidiCAT) assay [16] was employed for TG measurement using 5pM recombinant tissue factor (Innovin®), 16.7mM $CaCl_2$, 416.7 μM ZGGR-AMC (Bachem) in the absence and presence of 7nM recombinant TM. This TM concentration inhibits the ETP of a normal pooled plasma (NPP) by 50%. In each run the 500FG samples were tested besides an NPP, which was prepared by mixing the plasma of 116 healthy adult volunteers.

TG parameters in presence of TM are denoted as lagtime^{TM+}, time to peak (TTP $^{TM+}$), peak^{TM+} and ETP^{TM+}, respectively. The peak and ETP, both in absence and presence of TM, were normalized as percentage of that of NPP tested without TM. A normalized thrombomodulin sensitivity ratio (n-TMsr) was determined for the ETP and peak by dividing these values in presence and absence of TM, then normalized against the same ratio determined in NPP. A n-TMsr >1 means an increased resistance to TM compared with NPP.

Functional validation

Purified human PK, PKa (both from Enzyme_Research_Laboratories) or apolipoprotein A-IV (APOA4; from Sigma-Aldric) were titrated into NPP to designated concentrations before TG was initiated with TF (2, 5 or 15 pM), in the presence or absence of TM (1.5, 7 or 24 nM for different TF concentrations, respectively) or APC (0.8, 2.5 or 6 nM, respectively).

Genotyping

The genotype data of 500FG cohort were generated previously [15] using the single-nucleotide polymorphism (SNP) chip Illumina HumanOmniExpressExome-8 and was imputed to obtain approximately 7 million SNPs. The strands and variant identifiers were aligned to the reference Genome of The Netherlands (GoNL) dataset and other public datasets [17, 18] using Genotype Harmonizer v1 [19]. Data were phased and imputed by SHAPEIT2 v2.r64424 [20] and IMPUTE225 [21], respectively.

Statistics

Statistical analyses were performed with R and GraphPad Prism. Comparisons of the TG parameters between females and males were performed with the Mann-Whitney U test. A two-sided $p<0.05$ was considered statistical significance.

Inverse ranked based transformations were performed to normalize distribution in each TG trait before quantitative trait loci mapping were performed using an additive linear regression model with age and sex as covariates. Systematic inflation or deflation in test statistics over all loci was

assessed through the quantile-quantile (Q-Q) plot (**Supplemental Figure S1**) for TG parameters. A p-value $<5x10^{-8}$ was considered genome-wide significance and a p-value $<5x10^{-6}$ was defined as suggestive significance.

RESULTS

In total, 392 participants had both demographic data and TG parameters available, as shown in **Figure 1A**. The participants were young (median age 24), and 194 (49.5%) of them were female. Females and males were comparable in age (median & IQR: 23 [21-27] versus 24 [21-27], p=0.376) but the body mass index (BMI) was lower in the females (21.7 [20.5-23.7] versus 23 [21.6-24.6], p<0.001).

A

	Total (n=392)
Age, years	24 (21-27)
BMI, kg/m^2	22.4 (20.8-24.4)
Female, n	194 (49.5%)
PPP-TG in the absence of TM	
Lagtime, min	1.7 (1.4-2)
TTP, min	3.7 (3.3-4.3)
Peak, % of NPP	96.2 (83.9-113.8)
ETP, % of NPP	90.7 (80.4-106.9)
PPP-TG in the presence of TM	
Lagtime^{TM+}, min	1.7 (1.4-2)
TTP^{TM+}, min	3.4 (3.3-3.7)
Peak^{TM+}, % of NPP	66.2 (48.4-93.2)
ETP^{TM+}, % of NPP	53.4 (38.9-73.3)
TM sensitivity ratio	
nPeak-TMsr, ratio	1.1 (0.9-1.3)
nETP-TMsr, ratio	1.1 (0.8-1.4)

B

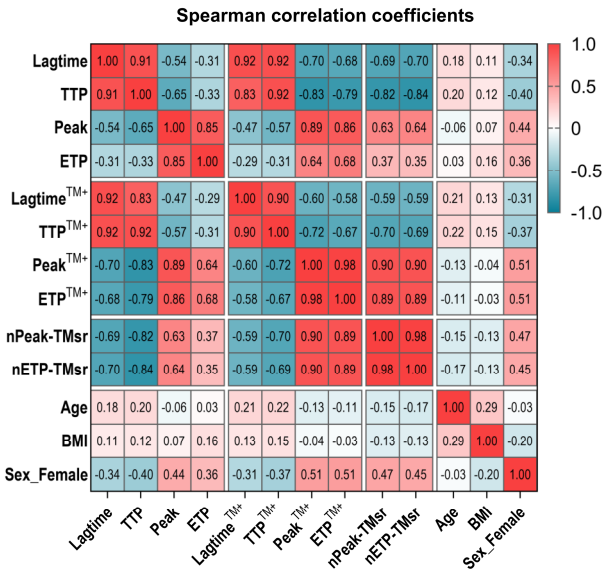

Figure 1. Demographic and thrombin generation parameters of the cohort. (A) Demographic and TG parameters of the cohort. Data are presented as number (percentage) or median (interquartile rang). TG parameters, both in the absence and presence of thrombomodulin (TM), were normalised as the percentage of the TG parameters of normal pool plasma (NPP) tested without TM in the same run. (B) Spearman correlation matrix of the TG parameters. The correlation coefficients are shown in the cells. Abbreviations: BMI, body mass index; TTP, time to peak; ETP, endogenous thrombin potential; nPeak-TMsr, normalised sensitivity ratio of peak to thrombomodulin; nETP-TMsr, normalised sensitivity ratio of peak to thrombomodulin.

Correlation coefficients between demographics, TG parameters and markers of the protein C pathway function (i.e. nETP-TMsr and nPeak-TMsr) are shown in **Figure 2**. The lagtime^{TM+} and TTP^{TM+} correlated strongly with their counterparts in absence of TM (spearman r > 0.83). The Peak^{TM+} and ETP^{TM+} correlated moderately to strongly with their counterparts in absence of TM (r > 0.64), and strongly with the normalized sensitivity ratio of peak and ETP (r > 0.89). Compared with male, females had stronger TG, as reflected by their shorter lagtime, higher peak and ETP; the TG of females were also more resistant to TM, as shown by the higher nETP-TMsr and nPeak-TMsr values (p<0.001 for all comparisons, **Supplemental Figure S2A-2C**). These differences between males and females were partially explained by the hormonal oral contraceptive use in females (n= 74 [38.7%]), which was previously shown to cause stronger TG and a reduced response to the anticoagulant effect of TM [22, 23].

Genome-wide significant loci for TG and the anticoagulant effect of TM

We explored SNPs that are associated with the TG parameters after adjusting for age and sex. A total of 327 samples were available for genetic analyses. As shown **Table 1** and **Figure 2**, a locus on chromosome 4 at the *KLKB1* gene (top SNP: rs4241819) was significantly associated with both the nETP-TMsr and nPeak-TMsr ($p < 5 \times 10^{-8}$ for both). The rs4241819-T allele (minor allele frequency 0.48) was associated with a higher nETP-TMsr value (β=0.36), i.e. a TM resistant prothrombotic tendency. Interestingly, the *KLKB1* gene has been found to be associated with venous thrombosis in several studies [24-26].

In addition, a locus at chromosome 9:23479590 (top SNP: rs404479) was significantly associated with TG capacity in presence of TM, i.e. ETP^{TM+} ($p=4.03 \times 10^{-8}$) (**Table 1** and **Figure 3**).

Furthermore, 8 suggestive loci were identified for TG parameters and protein C pathway function (**Supplemental Table S1**). For example, the ETP was associated positively with rs1767776-C on chromosome 6 and rs1985749-A on chromosome 17, whereas ETP $^{TM+}$ was negatively associated with rs610551-A on chromosome 9 ($p<6*10^{-7}$ for all correlation). However, none of these 8 loci have previously found to influence plasmatic coagulation.

Table 1. Genome-wide significant (p-value < 5 x 10^{-8}) loci for thrombin generation traits.

Trait	Top SNP	Gene	Chr	Position (bp)	Effect /Alternative allele	MAF	Beta	p-value
nETP-TMsr	rs4241819	*KLKB1*[a,b,c]	4	186235986	T/C	0.485	0.36	4.27×10^{-8}
nPeak-TMsr	rs4241819	*KLKB1*[a,b,c]	4	186235986	T/C	0.484	0.37	1.97×10^{-8}
ETP^{TM+}	rs404479	*RP11-315I14.2*[a]	9	23479588	T/G	0.433	0.46	4.03×10^{-8}

Note: a. Genes close to thrombin generation associated SNPs; b. eQTL effect of thrombin generation associated SNPs based on publicly available databases [39, 40]; c. Missense in LD variants (rs3733402). Abbreviations: SNP, single-nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; nETP-TMsr, normalized sensitivity ratio of endogenous thrombin potential to thrombomodulin; ETP^{TM+}, endogenous thrombin potential in presence of thrombomodulin.

We also explored the association between TG parameters and some previously known TG-related genes. A list of candidate genes was extracted from literatures [10-13, 27], in particular those that encoded coagulation factors, including *F5, HAAT, TFPI, FGA, F12, ORM1, F2, MYBPC3, PTPRJ* and *F10* genes. All variants located within 150kb of either end of the full-length transcript were examined to determine the strongest SNP association against each TG and protein C pathway function traits. Thirty-four SNPs associated with at least one TG parameters at significance levels of p <0.01 are shown in **Supplemental Figure S3**.

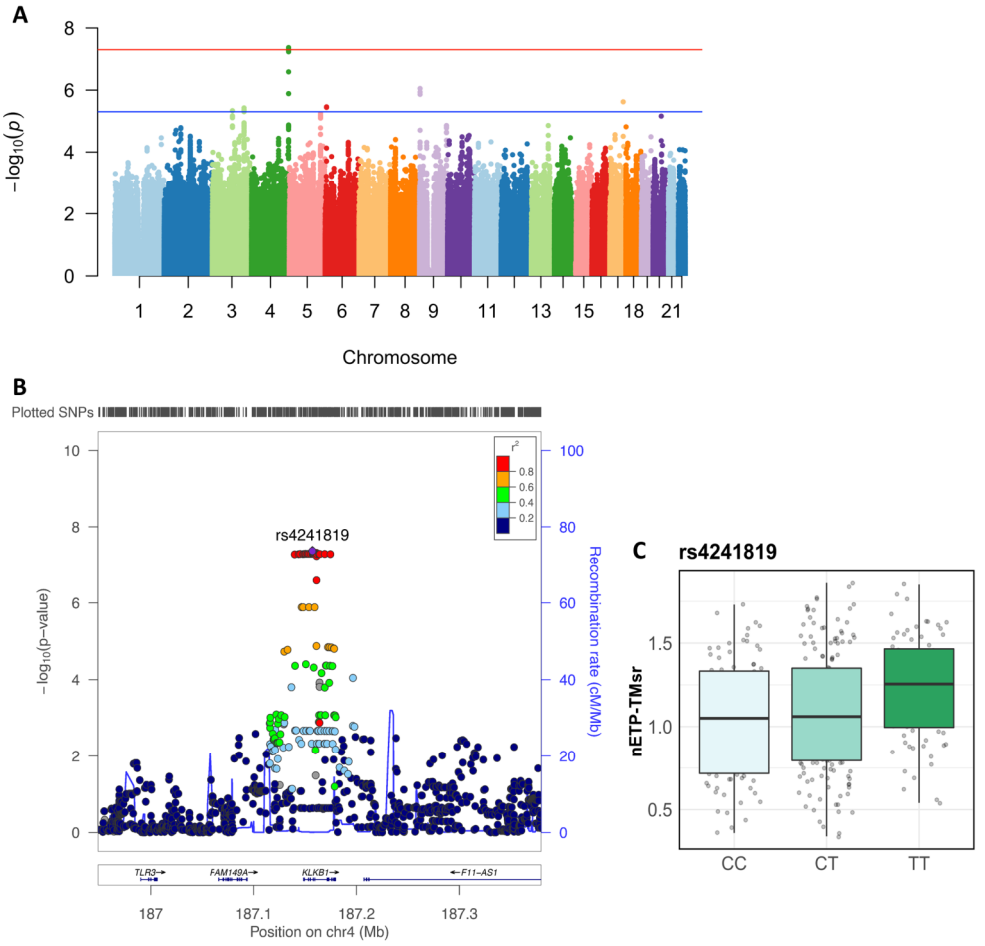

Figure 2. Genome-wide significant loci associated with the normalized sensitivity ratio of endogenous thrombin potential to thrombomodulin (nETP-TMsr). (A) Manhattan plot of SNPs associated with nETP-TMsr. The red line indicates the threshold for genome-wide significance (5×10^{-8}). (B) Regional association plot of genome-wide significant SNPs at chromosome 4. (C) Genotype stratified nETP-TMsr.

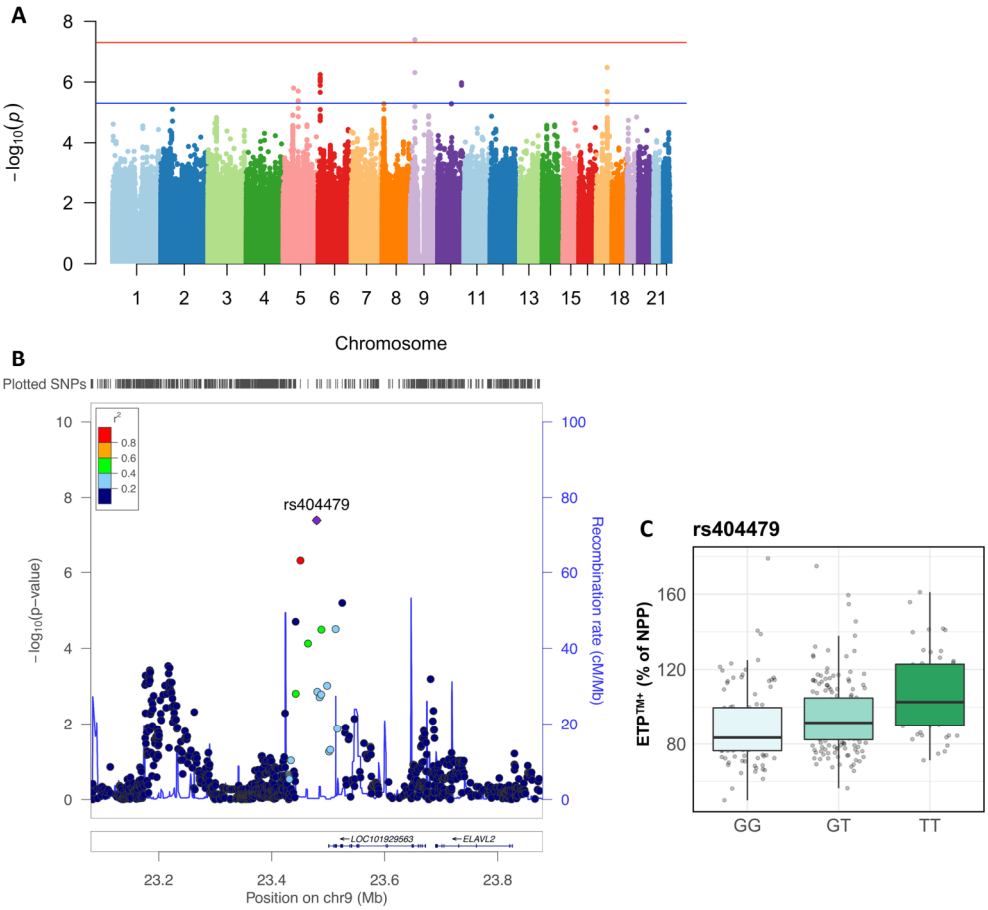

Figure 3. Genome-wide significant loci associated with ETP in presence of TM (ETP^{TM+}). (A) Manhattan plot of SNPs associated with ETP^{TM+}. The red line indicates the threshold for genome-wide significance (5×10^{-8}). (B) Regional association plot of genome-wide significant SNPs at chromosome 9. (C) Genotype stratified ETP^{TM+}. Abbreviations: ETP, endogenous thrombin potential; TM, thrombomodulin; NPP, normal pool plasma.

The anticoagulant effect of TM or APC was hardly affected by APOA4 supplementation

SNP rs4241819 was previously shown to influence the concentration of several plasma proteins including apolipoprotein A-IV (APOA4) [28, 29], a protein that was shown to influence arterial thrombosis risk [30, 31] and the rs4241919-C allele was related with an increased APOA4 concentration [28]. APOA4 circulates in blood at a concentration of approximately 150 µg/mL, in both a free form (75% of all) and another form that bounds to high density lipoprotein particles [32]. However, it was shown that the SNP rs4241819 only accounts for a very small

fraction (0.19 % to 0.44%) of the variations of APOA4 concentration ($\beta = 1.47 \pm 0.7$ μg/mL for the C allele) [28].

We tested the effect of APOA4 supplementation in a range between 0 to 40 μg/mL on the inhibitory effect of TM and APC in TG. As shown in **Supplemental Figure S4**, the inhibition rate of ETP by TM and APC remained largely unchanged in response to different APOA4 concentrations.

In addition, the rs42418189-T allele was also associated with decreased circulating concentrations of endothelin-2, protachykinin-1, and acidic leucine-rich nuclear phosphoprotein 32 family member B, as well as increased interleukin-2 concentration [29]. There are, however, no data on the relation of these proteins with the protein C pathway.

Kallikrein but not prekallikrein augmented the anticoagulant effect of TM and APC

The *KLKB1* gene encodes plasma kallikrein of the contact activation system. We first tested if concentrations of PK, the precursor of PKa, could affect TM- and APC- modified TG. As shown in **Supplemental Figure S5**, the supplementation of human PK at doses from 0 to 580 nM into NPP hardly affected the inhibitory effect of TM and APC on ETP.

We next explored if PKa supplementation could influence the anticoagulant effect of TM or APC in PPP-TG initiated by 5 pM TF. As shown in **Figure 4A**, the addition of PKa dose dependently enhanced the inhibition rate of ETP by TM and APC from 54.1% (no PKa addition) to 69.9% (when PKa was supplemented at 120 nM) ($p<0.001$). Meanwhile the ETP was not influenced by PKa addition. The inhibitory effect of APC on TG was similarly augmented by PKa addition.

We also tested the effect of PKa addition in TG reactions triggered by 2 and 15pM TF. For TG triggered by 2 pM TF, the ETP was increased from 1322 nM*min to 1472 nM*min when 120 nM PKa was added. This ETP-enhancing effect of PKa at low TF is expected because PKa can activate contact pathway factors FXII and FIX and enhance downstream TG. A stronger TG would also make it more difficult for a given concentration of TM or APC to deliver 50% inhibition on ETP. Indeed, the anticoagulant effect of TM or APC was not enhanced by supplemented PKa (**Figure 4B**). Contrarily, for TG triggered by 15 pM TF, the ETP inhibition by TM/APC increased dose dependently with the increasing PKa concentration added, from 54.1% with no PKa supplementation to 72.5% at 120 nM PKa (**Figure 4C**).

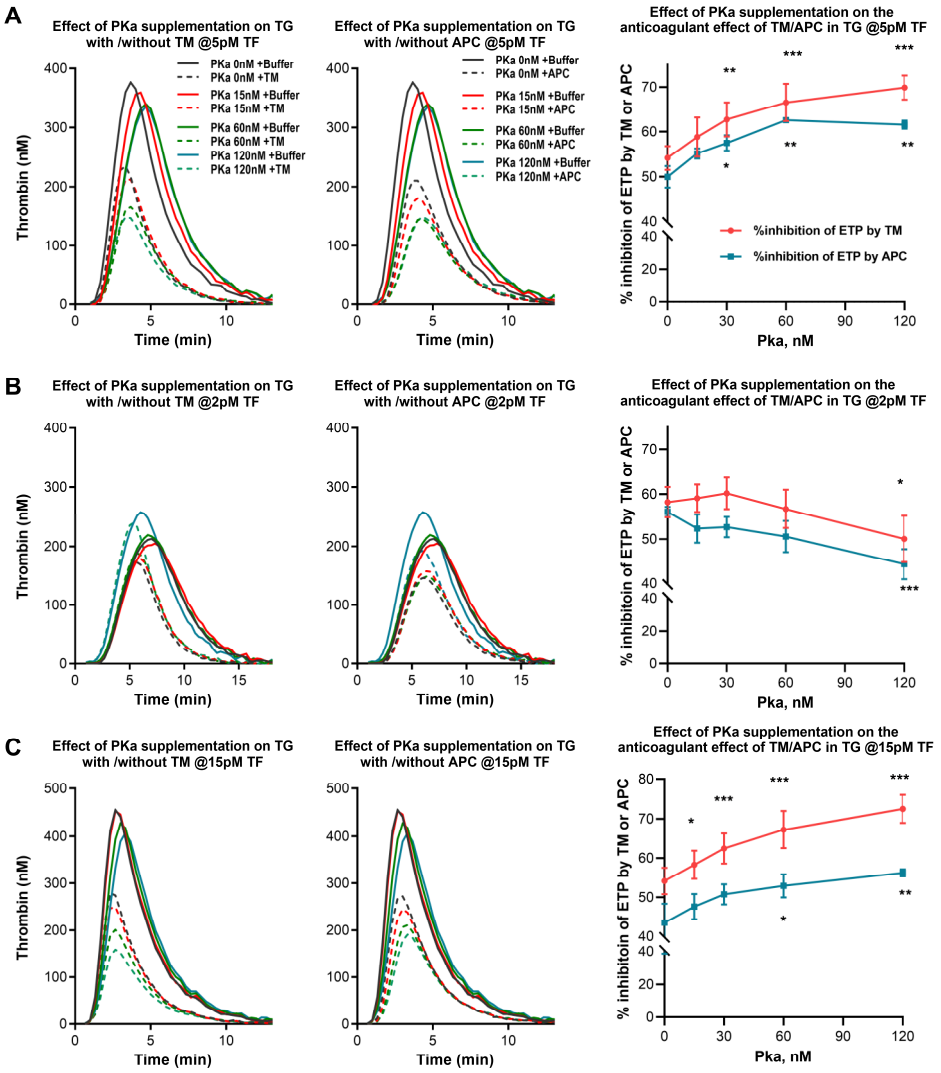

Figure 4. Effect of kallikrein (PKa) supplementation on the anticoagulant effect of thrombomodulin (TM) /activated protein C (APC) on thrombin generation (TG). TG was initiated with 5, 2 or 15 pM TF (shown in A, B and C, respectively), in the absence or presence of TM or APC in normal pooled plasma supplemented with varying doses of human PKa (at 0, 15, 30, 60 or 120 nM). Representative TG curves and a summary figure of the inhibitory effects of TM or APC on TG are shown in each row. Paired RM one-way ANOVA and Dunnett's multiple comparison test were used to compare conditions with and without PKa addition. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

DISCUSSION

In the present study, we found several genome-wide significant loci for TG parameters and protein C pathway function in a population of healthy individuals of Western-European origin. In particular, the *KLKB1* gene that encodes plasma PK was associated with the anticoagulant function of TM in TG. We provided a possible explanation for the observed genetic associations by demonstrating that in vitro supplementation of PKa augmented the anticoagulant effect of TM and APC in TG.

Besides our observation of a genome-wide association between the *KLKB1* gene (top SNP: rs4241819) and the protein C pathway function, several previous studies also found that this gene was associated with the risk of VTE [24-26]. Thus, our study provides a possible explanation that the *KLKB1* gene might mediate thrombotic risk through its influence on protein C pathway function. Our validation experiments further showed that in vitro supplementation of PKa augments the anticoagulant effect of TM and APC by approximately 30% in TG reactions triggered by both 5 and 15pM TF. A possible mechanism might be that kallikrein binds to protein C inhibitor (PCI) [33] and alleviate APC from inhibition by PCI. This mechanism might also protect people with excessive kallikrein activity (e.g. due to C1-inhibitor deficiency) from having excessive TG or clot formation.

We also found that PK supplementation hardly influence the inhibitory effect of TM/APC in TG, suggesting that the rs4241819-T allele is probably not related with a quantitative mutation. Interestingly, a recent study showed that a recombinant un-activatable form of PK can still activate FXII, suggesting that PK also possess proteolytic activity, although at a level 23 000-fold lower than PKa [34]. It is possible that the rs4241819-T allele results in a PK mutation that has a lower activation rate.

The SNP rs42418189 has also been shown to influence the plasma concentration of APOA4 [28], a lipoprotein that is involved in many physiological processes such as lipid absorption and metabolism, glucose homeostasis, anti-atherosclerosis, platelet aggregation and thrombosis [31]. It was shown that the SNP rs42418189 only accounts for a very small fraction (0.19 % to 0.44%) of the variations of APOA4 concentration ($\beta = 1.47 \pm 0.7$ μg/mL for the C allele) [28]. Our results showed that APOA4 supplementation at 40 μg/mL had negligible influence on the function of TM and APC in TG, therefore APOA4 does not explain the association between the above observed association.

We also found that the TG capacity in presence of TM (ETP^{TM+}) was significantly associated with a locus at chromosome 9: 23479588 (top SNP rs404497), which is transcribed to a long intergenic non-coding RNA. The effect of this variation on TG and coagulation requires further study.

A NPP was used to normalize the peak and ETP of the study subjects in each run, which is a major strength of our study approach. This approach could reduce the influence of inter-assay variations introduced by different measurement machines and different operators[35].

This study also has a few limitations that need to be delineated. Firstly, this study has a relatively small sample size and is mainly comprised of healthy donors, which limited our ability of identifying novel variants with low allele frequency. However, we were still able to show that the protein C pathway function was significantly associated with the *KLKB1* gene, a known VTE-related locus, implying that the PKa-protein C axis might be a common mediator of thrombosis in this population. This also reinforced that the TM-modified TG assay could serve as a useful tool to discover novel mutations related with the protein C pathway function. Secondly, the volunteers included in this study are mainly of Western-European background, so the applicability of these results in other ethnic origins is unknown. Thirdly, although our functional experiments with PKa supplementation in TG assay provided an explanation for the association between *KLKB1* gene variation and protein C pathway function, the exact mechanism remains unclear. It is likely that this variation leads to a qualitative mutation of PK, but further studies are required to assess the exact mechanism.

In conclusion, this study shows that kallikrein regulates the anticoagulant protein C pathway in TG, which may provide a novel mechanism for the previously observed association between the *KLKB1* gene and thrombosis.

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Author contributions:

M.R., Q.M., A.J., B.L., M.G.N., L.J. & V.K. conceptualized the study and provided essential resources. J.W.; J.K., M.J., M.R., P.G. & Q.M. did the measurements and/or analyzed data; N.V. performed genetic analyses. J.W., V.N. & M.R. wrote the manuscript with comments from all authors.

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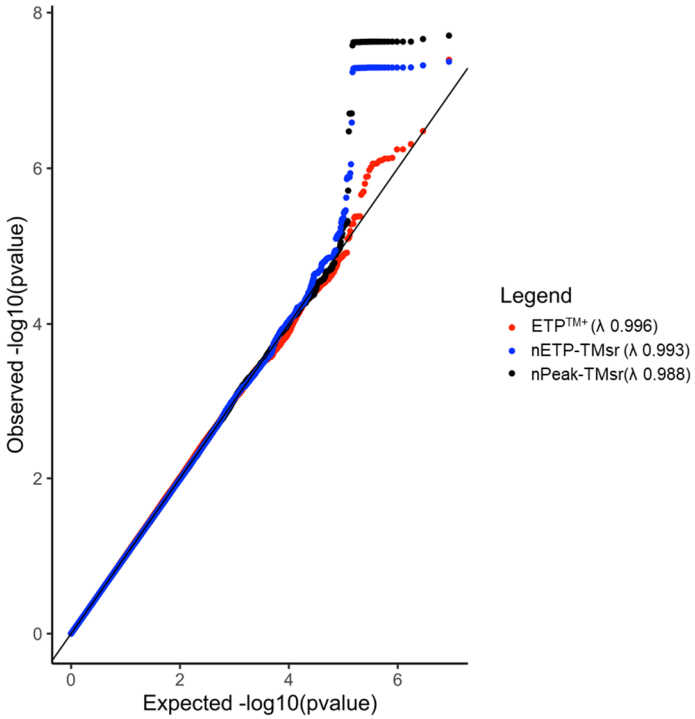
SUPPLEMENTAL INFORMATION

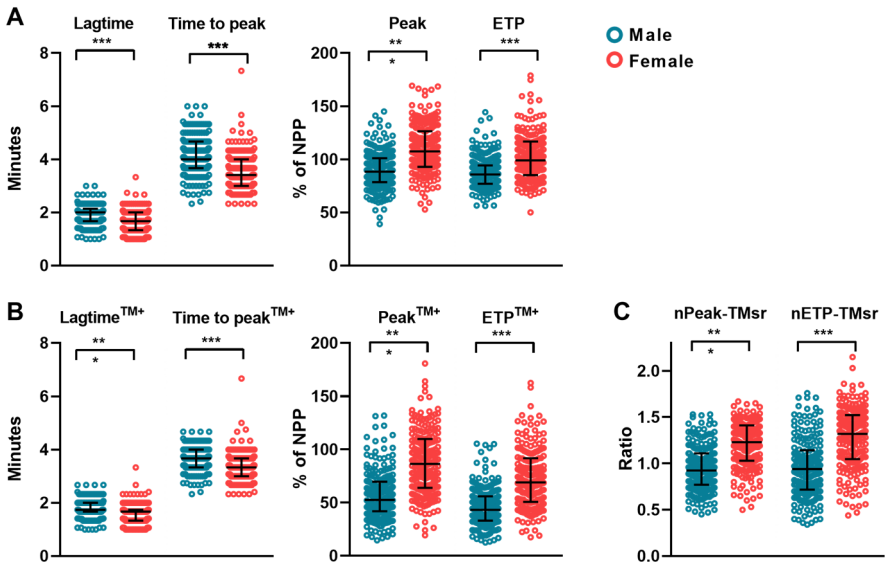
Supplemental Table S1. Suggestive significant loci (5×10^{-6} > p-value > 5×10^{-8}) for thrombin generation traits.

SNPs	Trait	Chr	Position (bp)	Effect /Alternative allele	MAF	P-value	Beta	Gene(s)
rs11616264	Lagtime	13	20898330	T/A	0.174	2.59×10^{-7}	0.55	*CRYL1*[a], *GJB6*[b]
rs1833710	Peak	5	147967746	A/G	0.11	1.88×10^{-7}	0.635	*HTR4*[a], *FBXO38*[c]
rs10199793	TTP	2	84233916	G/T	0.292	3.80×10^{-7}	-0.374	*RP11-315I14.2*[a]
rs1767776	ETP	6	10923852	C/G	0.187	5.70×10^{-7}	0.458	*SNORD112*[a]
rs1985749	ETP	17	56417002	A/G	0.323	3.32×10^{-7}	0.399	*RP5-1171I10.4*[a], *RAD51C*[c], *TRIM37*[c], *SUPT4H1*[c], *BZRAP1*[c], *MSX2P1*[c], *PRR11*[c], *MTMR4*[c], *SKA2*[c], *CTD-2510F5.4*[c], *SMG8*[c], *hsa-mir-142*[c], *MKS1*[c], *AC099850.1*[c]
rs11616264	Lagtime^{TM+}	13	20898330	T/A	0.174	9.59×10^{-7}	0.53	*CRYL1*[a]
rs610551	ETP $^{TM+}$	9	135968225	A/C	0.135	5.63×10^{-7}	-0.486	*snoU13*[a], *RALGDS*[b,c], *GBGT1*[c]
rs7045626	nETP-TMsr	9	2735203	A/G	0.357	8.88×10^{-7}	0.341	*KIAA0020*[a]

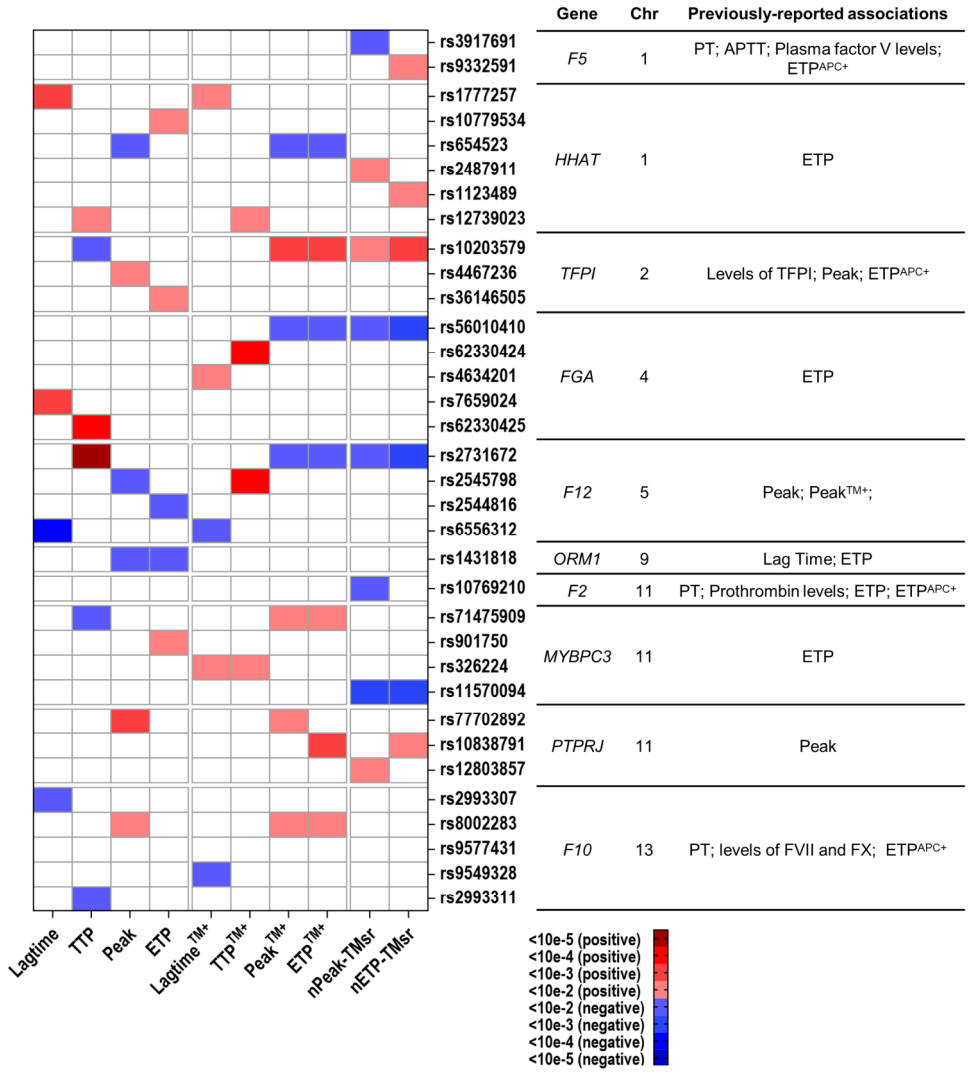
Note: a, Genes in close proximity to thrombin generation associated SNPs; b, Genes in close proximity to high LD variants ($r^2 \geq 0.8$); c, eQTL effect of thrombin generation associated SNPs based on publicly available databases. Abbreviations: SNP, single-nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; nETP-TMsr, normalized sensitivity ratio of endogenous thrombin potential to thrombomodulin; ETP TM+, endogenous thrombin potential in presence of thrombomodulin.



Supplemental Figure S1. Quantile-quantile (Q-Q) plot of the p values for ETP^{TM+}, nETP-TMsr and nPeak-TMsr. Inflation factors are shown in the graph and are all around 1. Abbreviations: ETP^{TM+}, endogenous thrombin potential in presence of thrombomodulin; nETP-TMsr, normalized sensitivity ratio of ETP to thrombomodulin; nPeak-TMsr, normalized sensitivity ratio of peak to thrombomodulin.

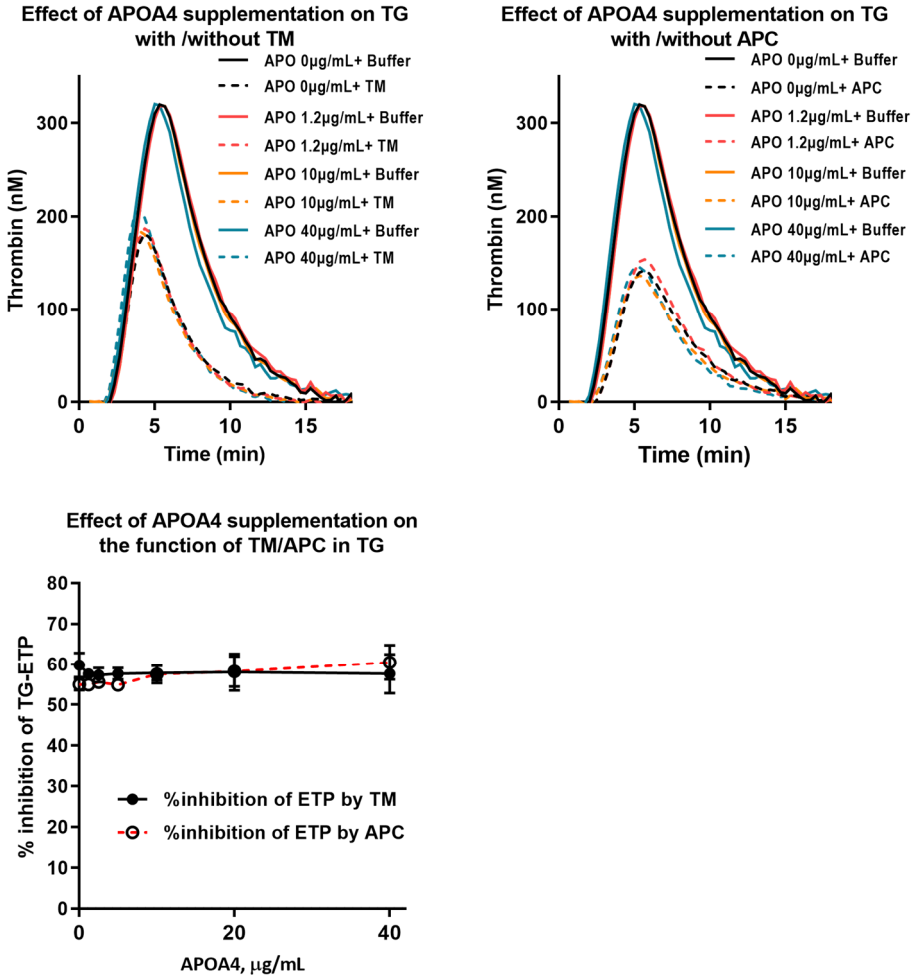


Supplemental Figure S2. Thrombin generation parameters in males (n=198) and in females (n=194). Bars in graph represents median and interquartile range. Comparisons between groups were done with the Mann-Whitney U test. ***, P<0.001. Abbreviations: NPP, normal pool plasma; ETP, endogenous thrombin potential; TM, thrombomodulin; nPeak-TMsr, normalized sensitivity ratio of peak to thrombomodulin; nETP-TMsr, normalized sensitivity ratio of ETP to thrombomodulin.

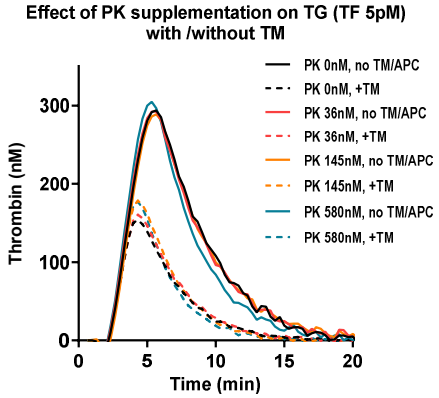


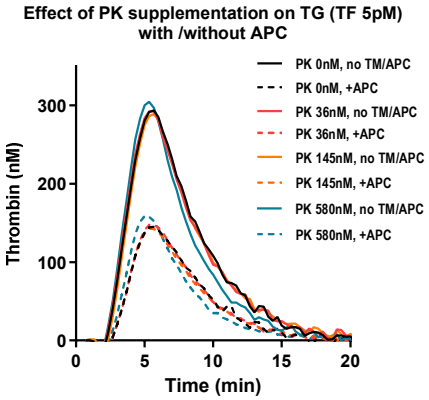
Gene	Chr	Previously-reported associations
F5	1	PT; APTT; Plasma factor V levels; ETP^{APC+}
HHAT	1	ETP
TFPI	2	Levels of TFPI; Peak; ETP^{APC+}
FGA	4	ETP
F12	5	Peak; Peak^{TM+};
ORM1	9	Lag Time; ETP
F2	11	PT; Prothrombin levels; ETP; ETP^{APC+}
MYBPC3	11	ETP
PTPRJ	11	Peak
F10	13	PT; levels of FVII and FX; ETP^{APC+}

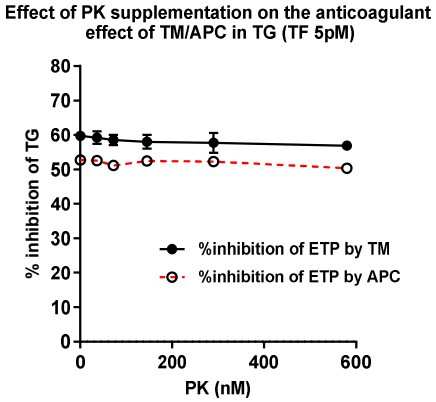
Supplemental Figure S3. Associations between single-nucleotide polymorphisms (SNPs) located in previously reported TG associated-candidate gene regions and TG parameters in our cohort. The colour legend indicates the range of p-values. Only the most significant SNP associated with each TG trait are shown (p-value <0.01). p-values were obtained from a linear regression model of TG parameters on genotype data. Abbreviations: TTP, time to peak; TM, thrombomodulin; ETP, endogenous thrombin potential; nPeak-TMsr, normalised sensitivity ratio of peak to thrombomodulin; nETP-TMsr, normalised sensitivity ratio of peak to thrombomodulin; PT, prothrombin time; APTT, activated partial thromboplastin time; APC, activated protein C; TFPI, tissue factor pathway inhibitor.



Supplemental Figure S4. Effect of apolipoprotein A-IV (APOA4) supplementation on the anticoagulant effect of thrombomodulin (TM) and activated protein C (APC) in thrombin generation (TG). TG was triggered with 5pM tissue factor (TF) in the presence or absence of 7nM TM (or 3nM APC) in normal pooled plasma supplemented with varying doses of APOA4 (1.2, 2.5, 5, 10, 20 or 40 µg/mL). Note that the normal concentration of APOA4 is approximately 150 µg/mL).







Supplemental Figure S5 Effect of prekallikrein (PK) supplementation on the anticoagulant effect of thrombomodulin (TM) /activated protein C (APC) on TG. TG was initiated with 5pM TF in the presence or absence of 7nM TM (or 3nM APC) in normal pooled plasma supplemented with varying doses of human PK (36, 72, 145, 290 or 580 nM). Note that the normal concentration of PK is approximately 580nM.

CHAPTER 8

Summary, general discussion and future perspectives

This thesis focuses on the innovation of thrombin generation (TG) assays and the application of TG assays in clinical and basic hemostasis research. In **chapters 2-4** we described the development and validation of a novel whole blood (WB)-TG assay for studying the involvement of blood cells in coagulation, as well as the exploration of the WB-TG capacity of cirrhotic patient using an optimized near patient WB-TG prototype. Furthermore, **in chapter 5** we reviewed currently available evidence on the added value of blood cells in TG testing. In **chapters 6 & 7** we used plasma-TG assay for the characterization of coagulation phenotype in people living with HIV (PLHIV) and explored the use of TG as an intermediate phenotype to discover novel coagulation-related genetic variants, respectively.

WB-TG ASSAY: INNOVATIONS

Numerous studies have shown that the capacity of generating thrombin is a critical determinant of blood coagulability[1]. The introduction of modern fluorogenic TG assays, such as the Calibrated Automated Thrombography (CAT)[2, 3], allows a comprehensive assessment of the interactions between pro- and anti-coagulant factors in platelet poor- (PPP) and platelet rich plasma (PRP) and achieved TG determination in an accurate and high throughput manner. To push TG testing closer to physiology and for practical considerations, the development of WB-TG assay is wanted. A fluorogenic WB-TG assay would be a nice option for high throughput applications, however it has been published that the erythrocytes in WB distort the fluorescence signal transmission, due to erythrocyte sedimentation during measurements.

A novel fluorogenic WB-TG assay

In **chapter 2** we presented a new assay that prevents erythrocyte sedimentation by continuously mixing the WB samples during the entire measurement [4]. This is achieved by (1) optimizing the setting of the Fluoroskan Ascent microplate fluorometer so that the 96-well plate moves continuously during the entire measurement course, without noticeable stop between two rounds of fluorescence reading, and (2) choosing a 96 well plate with round-cornered wells to allow better mixing effect.

Previously reported fluorogenic methods for WB-TG measurement suffered from high imprecision[5] or strong interference of contact activation[6]. In contrast, our novel assay demonstrated good intra- and inter-assay reproducibility and was less affected by contact activation, as well as required less handling steps compared with the filter paper-based WB-TG assay[6]. Validation of this assay in 119 healthy donors showed that the inter-individual variation of WB-TG was shown to be comparable to that of plasma-TG, suggesting the introduction of blood cells did not add further variation. Furthermore, previously identified prothrombotic risk factors (i.e., older age[7] and oral contraceptive use[8, 9]) influenced our novel WB-TG assay. The reference ranges of the WB-TG parameters were determined to detect abnormal WB-TG parameters in different patent groups.

A limitation of this assay is that a multichannel pipetting step is still needed to simultaneously activate coagulation in multiple wells, thereby requiring pipetting skills. Of note, due to the

higher variations in the tail part of fluorescence curve, we were restricted to only calculate the area under the TG curve until the thrombin peak (termed ETPp) using a method that requires the fitting of a Chapman-Richard growth (CRG) function[10, 11] on fluorescence data. This calculation method has the advantage that the measurement time can be reduced from 50 to 20 minutes, but this may also prevent the accurate calculation of ETPp in samples that have slow TG, e.g. the use of high dose of anticoagulants. However, after some recent optimization steps, an acceptable noise in the tail part of the WB-TG curve can be obtained, enabling us to calculate a complete WB-TG curve (see below for more details).

An optimized near patient WB-TG assay

The WB-TG test does not require centrifugation steps for plasma preparation and has therefore shorter turn-around time and avoids some pre-analytical variations introduced during plasma preparation. These aspects also make it possible for near patient testing outside a specialized laboratory environment. Viscoelastic tests including thromboelastography (TEG) and rotational thromboelastometry (ROTEM) are also near patient hemostatic tests and have been extensively explored in the field of thrombosis and hemostasis. The viscoelastic tests have shown usefulness for the management of bleeding complications during surgery and in acute trauma care, especially in reducing the amount of blood product transfusion[12]. However, assessing fibrin clot formation only reflects the procoagulant effect of thrombin, whereas incomplete information is given on the anticoagulant pathways, particularly the anticoagulant effect of thrombin through the protein C pathway. On the contrary, WB-TG covers the entire course of thrombin activation and inactivation, including the function of the protein C pathway if thrombomodulin is supplemented, thereby making it more suitable for predicting thrombophilia[4, 13].

In **chapter 3**, we described an optimized near patient WB-TG assay using a previously described miniaturized fluorometer[14] and a newly modified microfluidic chip[13]. The previous chip, which was made from methyl methacrylate-acrylonitrile-butadiene-styrene (MABS)[14], suffered from occasional fluid leakage. We adopted a new design for the chip, which consisted of two pieces of polydimethylsiloxane (PDMS) with a porous matrix disc in between. After treatment in a plasma cleaner, permanent bonds form between the two PDMS parts thereby preventing leakage. This improved assay has an acceptable reproducibility: the inter-assay variations of all TG parameters were less than 15%. Of note, this assay can only measure one sample per run, a separate run needs to be done for calibration. The mixing of a blood sample with reagents (substrate, tissue factor etc.) was still done outside the device, which is not an ideal option.

THE INFLUENCE OF BLOOD CELLS ON TG

The influence of blood cells on TG

Compared with plasma-TG, WB-TG includes all circulating blood cells, making it a good tool to study the involvement of blood cells in thrombosis and bleeding. PRP-TG reflects the impact of platelet on TG, including granule content release[15] and interaction with coagulation factors[16]/autoantibodies[17] (see **chapter 5** for more detail[18]).

By applying our newly developed WB-TG assay, we were able to show that increasing erythrocyte counts dose-dependently augmented the peak of WB-TG in reconstituted blood[4], which is in line with previous studies using a subsampling TG method[19]. Importantly, we found that erythrocyte count enhanced the thrombin peak even in presence of high numbers of platelets in reconstituted blood[4]. This stimulating effect of erythrocytes was also found in our study in a group of 119 healthy donors[4] and cirrhotic patients[13], in which erythrocyte count was positively correlated with the peak height. This stimulating effect of erythrocytes may stem from the exposure of phosphatidylserine in a subpopulation (approximately 0.5%) of erythrocytes[19], or through the ability of erythrocyte membrane to activate the contact pathway[20-22], or via an indirect manner through enhancing platelet activation[23, 24].

In addition, we observed that higher erythrocyte count was associated with a slightly longer lagtime of WB-TG in both reconstituted blood and healthy donors[4], whereas the inclusion of platelets was essential for a fast onset of TG in reconstituted blood, suggesting a different role of platelets and erythrocytes in TG initiation. Platelets are equipped with a variety of surface receptors for coagulation factors and can express phosphatidylserine when they are activated by small amount of thrombin generated during the initiation phase of TG. These receptors and phosphatidylserine serve as a good surface for the localization of coagulation factors on platelet surface and facilitate the amplification of TG reactions. On the contrary, erythrocytes may hinder the initiation of TG due to steric hindrance induced by their big size (10 times larger in mean cell volume than platelets) and high numbers in circulation (15 times higher number than platelets). However, erythrocytes may contribute to the propagation of TG after they are trapped in a fibrin clot. Taken together, our results suggest that platelets are essential for a fast onset of TG in WB whereas erythrocytes influence the velocity of TG[18].

In addition, physiological coagulation is likely to be also affected by interactions between different blood cells and the WB-TG assay might be a tool to study the influence of these interactions. For example, previous studies have shown that blockage of erythrocyte-platelet interactions, either by blocking the interaction of platelet integrin αIIbβ3 with erythrocyte receptor intercellular adhesion molecule (ICAM-4)[24] or by preventing the binding of platelet Fas ligand with erythrocyte Fas receptor[23], induced reductions in thrombus formation or WB-TG. It would be interesting to test the effect of other cell-cell interactions on WB-TG because this might be a useful approach to identify novel anticoagulant strategies.

The differential effects of erythrocyte/platelet count on the rate and capacity of WB-TG

In both a healthy population and cirrhotic patients we found that the counts of erythrocytes and platelets exhibited differential effects on the rate and capacity of TG[4, 13]. Specifically the endogenous thrombin potential until peak (ETPp) plateaued once the platelet count reached a certain level (approximately $100*10^9$ platelets/L), but the peak still increased with further ascending platelet counts. Similar trends were observed with erythrocyte counts[4]. Currently many studies regard ETP as the most important parameter of TG, but our observation here suggests that both the peak and ETP should be reported in future studies.

The influence of erythrocytes on the anticoagulant function of the protein C pathway in TG

The protein C pathway exert its anticoagulant function through proteolytically inactivating factors Va and VIIIa by activated protein C (APC)[25]. A modification of the TG assay by supplementing soluble thrombomodulin (TM) or snake venom Protac enables the activation of intrinsic protein C and allows a more complete profiling of the coagulation system[2]. The supplementation of APC into the TG assay makes the assay sensitive for factor V Leiden and prothrombin G20210A mutation, to protein S deficiency, hormone replacement therapy, oral contraceptive use and pregnancy[8, 26, 27].

In **chapter 4** we found a new role of erythrocytes in regulating the protein C system. Our results showed that the anticoagulant effects of APC and TM were approximately 3 times weaker in WB- than in PRP-TG. Both in blood drawn from a heathy population and PPP samples reconstituted with varying erythrocyte counts, a higher erythrocyte count was related with a weaker effect of TM and APC. These results indicate that erythrocytes impair the anticoagulant function of APC but not the generation of APC. Previous studies have shown that platelet activation induces APC resistance[28] by providing a membrane surface that delays cofactor inactivation[29] and by releasing their α-granule content containing platelet factor 4 (PF4) that inhibits FVa cleavage by APC[30]. The mechanism underlying the adverse effect of erythrocytes on APC's anticoagulant effect is still unclear, but it is not likely through an indirect, platelet dependent manner because erythrocytes added into PPP, in the absence of platelets, also diminished the anticoagulant effect of APC on TG. The phospholipid (PL) composition and erythrocyte-induced steric hindrance may play a role. Previous studies have shown that the activity of APC can be enhanced by certain PLs, in particular phosphatidylethanolamine (PE)[31], which exist abundantly (20% in mole) in synthetic PL. Whereas a recent study that combined phospholipase digestion with mass spectrometric lipidomics found that PE only account for 5.8% of the PL composition in the outer leaflet of erythrocytes[32]. Our observation that the supplementation of small sized synthetic PL vesicles augmented the anticoagulant effect of TM and APC in WB-TG may also suggest that erythrocytes-induced spatial hindrance hampers the function of APC in WB.

Based on our finding that erythrocytes influence the function of APC, it is very likely that the WB of individuals with polycythemia who have abnormally high counts of erythrocytes is more resistant to APC compared to people with normal erythrocyte counts. Therefore, our finding might have increased our insight regarding the mechanism of the high thrombotic risk in polycythemia patients[33] as classical coagulation tests, mostly tested in plasma, may only give an incomplete picture on the regulation of coagulation. In addition, our findings may also provide an additional explanation for the increased thrombotic risk associated with erythrocyte transfusion[34].

THE APPLICATIONS OF TG IN FUNDAMENTAL AND CLINICAL RESEARCH

WB-TG in cirrhotic patients

Because of the distortions of both the pro- and anticoagulant pathways, patients with liver disease have complex coagulation profiles[35, 36] that cannot be accurately captured by traditional coagulation tests such as prothrombin time (PT) and the activated partial thromboplastin time (APTT), as they only reflect the impairment in the pro-coagulant pathways and are mostly insensitive to the changes in the anticoagulant pathway[1, 37]. In **chapter 3**, we explored the WB-TG profile of a group of cirrhotic patients for the first time and found normal WB-TG capacity (ETPp) but a lower TG rate (peak height) in cirrhotic patients, both in the absence and presence of thrombomodulin, suggesting normal- to hypocoagulability. Meanwhile, we also showed that TM-modified PPP-TG of cirrhotic patients had both elevated ETP and peak, suggesting hypercoagulability in cirrhosis. Our PPP-TG results were in line with most previous studies conducted in this patient category[37-39] and reinforced the notion that cirrhotic patients do not necessarily acquire a bleeding tendency as indicated by their often prolonged PT and APTT, but rather have a fragile rebalanced coagulation system[40-42]. The normal ETPp of WB-TG copes well with the concept of rebalanced coagulation, but the clinical indication of the reduced peak is unknown and is probably related with the moderately reduced counts of platelets and erythrocytes, which, as described above, could have differentially influenced the ETPp and peak and induced the discrepancy between these two parameters.

The PPP-TG results in our study also indicated a TM-resistant phenotype in cirrhotic patients, which has been repeatedly shown in previous studies and was believed to be a consequence of reduced plasma protein C level and increased FVIII concentration[43, 44]. A reduced response to TM was also seen in the WB-TG of cirrhotic patients compared with controls[13]. However, the inhibitory effect of TM on TG was drastically lower in WB than in PPP, most probably due to the negative effect of erythrocytes as described above. Overall, the information on the function of the protein C pathway in the WB of cirrhosis is rather limited. Further studies with larger sample size and follow up data will be needed to determine which parameter of WB-TG is more useful in guiding replacement therapy/anticoagulation in these patients.

PPP-TG in People living with HIV (PLHIV)

In **chapter 6** we assessed the PPP-TG profile of 208 PLHIV on combination antiretroviral treatment (cART) and compared to 56 uninfected healthy controls. The introduction of combined antiretroviral treatment (cART) has nearly normalized the life expectancy of PLHIV; however it is not able to completely prevent immune activation and the risk of venous thrombosis is still elevated in these individuals [45-48]. Indeed, inflammation was increased in PLHIV, as reflected by the increased sCD14, high-sensitive c-reactive protein and sCD163, and was associated with the concentration of in vivo coagulation marker D-dimer, in line with previous studies [49, 50] . However, these inflammation markers were not associated with ex vivo plasmatic coagulation potential ETP, suggesting that increased D-dimer are primarily influenced

by increased provoking signals for coagulation, such as endothelial activation and inflammation during HIV infection.

There is an ongoing debate on the effect of cARTs, especially abacavir, on the risk of cardiovascular disease [51-54]. We showed that abacavir-use was associated with a procoagulant TG profile compared to non-abacavir regimens, even after adjustment for age, sex and inflammation. This result is in contradict with a previous study by Jong et al that showed no difference in TG for abacavir-use, but only with a limited sample size of 27 individuals in the abacavir-group[55].

A subgroup analysis of non-abacavir users showed a slight decrease in TG in PLHIV compared to uninfected controls, in line with the only other report that compared PLHIV on cART with healthy controls[56]. Consumption of clotting factors could be part of the explanation, as in our study the prothrombin concentrations were decreased with a concurrent increase in D-dimer in the total PLHIV group. In this study we did not have data to link TG profiles with clinical outcome but previous studies have linked a higher in silico TG with higher mortality in PLHIV[57]. However, actual measurement of thrombin formation using the CAT method is more precise than the modelling of the *in-silico* TG method, because CAT also includes possible unknown confounders such as cART-use or other unknown factors.

This study is the first that has used TM-modified TG to the function of the protein C pathway in PLHIV. The ETP and peak of PLHIV were more responsive to TM compared to healthy controls, indicating a better function of the protein C pathway. Although protein C levels were not measured, the protein S levels were comparable in PLHIV and controls, in line with previous studies[55, 58]. Therefore, the hyper-responsiveness in PLHIV might exist because of the weak TG in those individuals. A previous study by Schnell et al also observed a hyper-response to APC in both PLHIV on cART and those not[56], however Jong et al showed that PLHIV on cART, both with and without abacavir, were more resistant to APC compared with a reference range although both the protein C and S levels were within the normal range[55, 58].

The cross-sectional design of our study does not allow to draw causal inferences about the effect of abacavir treatment. Furthermore, we cannot exclude the possibility that lamivudine and tenofovir difumarate have an inhibitory effect on TG because these drugs were the most prescribed alternative to abacavir. Future studies are warranted to verify these associations.

TG as an intermediate phenotype to identify novel coagulation-related genetic mutations

Previous studies demonstrated that TG profiles could serve as a useful intermediate phenotype to discover genetic variations related with venous thromboembolism[59-62]. Not only were several established thrombophilic mutations (including FV Leiden and FII G20210A) related to enhanced TG or APC-modified TG[60, 61], one study also successfully used the TG profile in the discovery of the *ORM1* gene as a significant locus with lag time variability [62].

In **chapter 7** we measured TM-free and TM-modified TG in a group of healthy individuals of western-European background and used these TG profiles to discover novel genetic variations

related with coagulation. The TM-modified TG makes the assay dependent on endogenous protein C, therefore reflects a more complete picture of physiological coagulation, and may allow the discovery of new quantitative/qualitative mutations related to the protein C pathway function. The main finding was that the *KLKB1* gene that encodes plasma kallikrein was associated with the anticoagulant function of TM in TG. Interestingly, several previous studies have shown that this locus is associated with venous thrombosis risk[63-65]. Thus, our study provides an explanation that the *KLKB1* gene might mediate thrombosis risk through its influence on protein C pathway function.

PKa is a component of the contact coagulation system. Prekallikrein, the zymogen of PKa, circulates in plasma at approximately 580 nM in complex with high molecular kininogen. PK is activated to PKa by FXIIa, which then lead to the reciprocal activation of both enzymes and leads to the activation of FXI and downstream TG[66]. Recently a novel function of PKa of directly activating FIX was discovered by several groups[20, 67, 68] but by now no mechanism has been found for PKa in regulating the protein C pathway. Our functional experiments further showed that in vitro supplementation of kallikrein (PKa) augments the anticoagulant function of TM and APC in TG. A possible mechanism might be that kallikrein binds to protein C inhibitor (PCI) [69] and alleviate APC from inhibition by PCI. We also showed that PK supplementation hardly influence the inhibitory effect of TM/APC in TG, suggesting that the rs4241819-T allele is probably not related with a quantitative mutation. Interestingly, a recent study showed that a recombinant un-activatable form of PK can still activate FXII, suggesting that PK also possess proteolytic activity, although at a level 23 000-fold lower than PKa[70]. It is possible that the rs4241819-T allele results in a PK mutation that has a lower activation rate.

The main limitation of our study is the relatively small sample size (n=392), which hamper the discovery of novel mutations with lower allele frequency. However, we were still able to show that the protein C pathway function was significantly associated with the VTE-associated *KLKB1* gene, implying that this gene might be one of the common mediators of thrombosis in the general Caucasian population. This also reinforced that the TM-modified TG assay could serve as a useful tool to discover novel mutations related to the protein C pathway function. The use of a cell-dependent TG, e.g. WB-TG, may further facilitate the identification of variations that are related with platelet/erythrocyte/leukocyte function and may provide new anticoagulation targets.

FUTURE WORK TO BRING WB-TG TO THE CLINICS

At the moment, TG assays are performed under near-static conditions, so the impact of blood flow on the interactions between platelets and VWF or other coagulation proteins are lacking. Although the addition of TM can partly resemble the anticoagulant function of endothelial cells, they also synthesize and release other coagulation proteins, most notably TFPI and VWF.

Despite the above limitations, various studies have shown that TG still provides a very good indication of one's coagulation potential and is a promising diagnostic tool. To make the WB-

TG suitable for clinical use, several improvements regarding assay automation and standardization of pre-analytical and analytical conditions are needed. Clinical studies are also needed to establish the clinical relevance of WB-TG parameters.

Optimization of the WB-TG assay for better automation

Our recently developed WB-TG assay[4] makes an accurate TG measurement in WB significantly easier compared with previous methods[6]. However, several limitations still exist that makes it challenging for clinicians to operate. First, this assay still uses multichannel pipets to (1) allow simultaneous triggering of coagulation in multiple reaction wells and (2) mix the reaction mixture to a homogenous solution. Therefore, a proper skilled technician is required. This manual mixing technique is also not ideal for high throughput measurements in large clinical studies. Secondly, to allow adequate fluorescence signal output on a Fluoroskan Ascent microplate fluorometer, a rhodamine-based substrate ($[ZGGR]_2$-Rhodamine) that has a higher fluorescence yield is used in our WB-TG assay. This substate is, however, is not as widely available as the ZGGR-AMC that is used in plasma-CAT and has a more complex reaction property due to the presence of two peptide chains.

Recently, through several optimizations in the assay setup, we are close to solve the above-mentioned problems and make the operation difficulty of WB-TG comparable to that of the classic plasma-based CAT. Firstly, multichannel pipetting can be replaced by (1) the use of the dispenser to simultaneously add substrate/$CaCl_2$ solution (i.e. the FluCa) into WB and, (2) the use of an intensive mixing step to mix the above mixture to a homogeneous solution. An intensive mixing step is required because the viscosity of WB is much higher compared to plasma, mostly due to the presence of large numbers of erythrocytes. Secondly, a background shaking is used to mix the reaction solution between two rounds of fluorescence readings to prevent erythrocyte sedimentation. Lastly, changing the excitation/emission filter pair from the current 390nm/460nm to 355nm/460nm results in a 3.5-fold increase in fluorescence intensity, which significantly improved the fluorescence signal output of ZGGR-AMC in WB-TG. This optimized WB-TG assay maintained a good reproducibility. This was shown by that the intra-assay CVs were less than 10%. More investigations on inter-assay and inter-operator reproducibilities are required to establish its performance. To bring WB-TG into the clinic, further innovations of fully automation are needed. An ideal upgradation would use standardized reagents, internal quality controls and reference blood sample, similar to the fully automated PPP-TG system ST-Genesia[71-73].

Standardization of assay conditions and establishment of clinical relevance

The lack of standardization on pre-analytical variables and analytical protocols, as well as the absence of standardized reagents and reference sample greatly hampers the evaluation of its clinical value in large scale studies. For example, the use of CTI was shown to improve the reproducibility of plasma-TG when the assay was triggered with low concentration of TF [74, 75]. WB-TG is typically triggered with low amounts of TF (0.5 or 1 pM) [4, 6] so that coagulation is dependent on physiological PL provided by blood cells and on feedback loops. But whether the use of CTI would also impact the reproducibility of the WB-TG assay requires further

assessment. The assay trigger should also be optimized for different settings; for instance, a trigger that contains low or even no TF maybe necessary to reflect the procoagulant effect of TF/phosphatidylserine on cancer cells or microparticles. Whereas for the characterization of effects of novel oral anticoagulants on WB-TG a high TF concentration should be used for obtaining faster results.

In addition, it is necessary to have a standard sample for the normalization of WB-TG result to allow comparison of results between laboratories. However, this is challenging because PRP or WB cannot be stored for a long time. Long storage-induced platelet storage lesion induces platelet activation and aggregate formation in stored WB and platelet concentrate.[76] Although several efforts have shown to increase platelet viability during storage[77], their effect is quite limited and more innovative ideals are needed.

Lastly, more studies are needed to determine the clinical usefulness of the WB-TG parameters. Further mechanistic and clinical studies are needed to determine which parameter(s), or the integration of parameters into a score, are most useful.

CONCLUSION AND PROSPECTS

In conclusion, in this thesis we presented innovative WB-TG assays that allow direct measurements of the influence of blood cells on TG. Although more studies on the standardization and clinical relevance of TG assays are needed, our preliminary results suggest that plasma- and WB-TG assays have promising applications in fundamental and clinical research.

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APENDICES

IMPACT

Cardiovascular disease (CVD) represents the number one cause of death globally, claiming an estimated 17·8 million deaths in 2017 [1]. Especially, the threat of thrombosis is increasing as the global population is aging. Dysfunction of the blood coagulation system is one of the major causes of CVD. New developments in coagulation tests will give better insights in the involvement of coagulation in CVD[2].

Thrombin generation (TG) is a test to measure the capacity of blood to form thrombin, the key enzyme in the blood coagulation system. TG gives a global overview of the coagulation capacity of an individual, thus may give insight in the risk of bleeding/thrombosis. Furthermore, TG may be a tool to guide patients on anticoagulant treatment. Although TG is the most complete plasma based coagulation assay [3], it does not involve the interplay between coagulation and blood cells [4]. The main goal of this thesis was to innovate/improve whole blood (WB) -TG assays to study the contribution of blood cells to the regulation of TG.

Key contributions of this thesis:

We developed a novel fluorogenic WB-TG assay with good reproducibility. This assay is a major step forward compared to previous WB-TG assays that had a poor reproducibility [5], or were prone to contact activation induced by the filter paper in the assay [6]. Platelet and erythrocyte counts, as well as platelet (in)activation were found to be crucial determinants of WB-TG, supporting our hypothesis that our WB-TG assay rightfully includes the influence of blood cells on coagulation. A strong enhancing effect of erythrocytes on the velocity of TG was observed, even in the presence of high platelet numbers. We also explored the performance of this assay in a healthy population and studied it in relation to age, gender, oral contraceptive use and blood cell count, which could serve as useful references for future studies.

We studied WB-TG profiles of cirrhotic patients with an optimized near patient assay and found that the TG capacity of these patients was comparable to that of normal people, suggesting a normal coagulability, in line with the widely accepted concept of rebalanced hemostasis in these patients [7]. Interestingly, the WB-TG velocity was slower in cirrhotic patients despite an intact WB-TG capacity. This observation might be explained by our previous results in reconstituted blood that counts of erythrocytes and platelets impact the TG velocity and capacity differently, thus a mild decrease of platelet and erythrocyte counts in cirrhosis only impaired the velocity of WB-TG but not the total capacity. The balance of the coagulation system in cirrhotic patients is very fragile and may easily tip towards a bleeding or a thrombosis phenotype. By using WB-TG a likely better representation of the in vivo situation is established, and WB-TG might be the assay of choice to study influences of therapy on fragile equilibria such as with liver cirrhosis and to predict their bleeding or thrombotic risk.

We found an inhibiting role of erythrocytes on the anticoagulant function of activated protein C in WB-TG. The protein C system is an important anticoagulant pathway and impairment of this

system is a common cause of thrombosis [8]. Higher erythrocyte count was related with a reduced anticoagulant effect of active protein C and thrombomodulin in WB-TG, both in a healthy population and in reconstituted blood samples. This effect was not dependent on platelets and was likely related with the phospholipid composition of erythrocytes. The inhibiting role of erythrocytes on APC function, combined with the observation of the enhancing effect of erythrocytes on WB-TG velocity, might provide a possible explanation for the increased thrombotic risk related with increased erythrocyte counts, for example polycythemia and erythrocyte infusion [9, 10].

We studied the plasmatic coagulability of HIV-infected individuals on combined antiretroviral therapy and found that the plasma TG capacity of these individuals was lower than healthy controls. This observation, together with their reduced prothrombin levels and increased markers of inflammation and endothelial activation, suggest that the increased thrombotic risk of these individuals was not due to hypercoagulability and was most likely related to increased stimulation of coagulation by endothelial activation and inflammation during HIV infection. We also found that abacavir-use was associated with a prothrombotic TG profile compared to non-abacavir regimens, irrespective of age, sex and inflammation, thus providing new data for the debated thrombotic effect of abacavir [11].

We used TG as an intermediate coagulation phenotype in a genome wide association study and discovered that the *KLKB1* gene was related to the anticoagulant function of the protein C system. Functional experiments showed that in vitro supplementation of kallikrein augments the anticoagulant function of TM and APC in TG. This provides a possible mechanism for the previously observed association between the *KLKB1* gene and thrombosis [12, 13]. This also reinforces that the TM-modified TG assay could serve as a tool to discover novel mutations related to the protein C pathway.

Conclusion &prospects

In this thesis we present new tools for improved WB-TG measurements and data on the utility of TG assays in both fundamental and clinical research. Our data support the view that blood cells play important roles in the regulation of coagulation and suggest that WB tests provide additional insights into plasma tests. WB-TG is still in its infancy and additional research is needed to improve its standardization and establish its clinical utility.

WB-TG does not require a plasma preparation step, thus the research described in this thesis could aid to the development of a point-of-care (POC) TG test for coagulation assessment in places without a specialized hematology laboratory. Such a POC-TG test could allow people to have a more detailed and timely overview of their coagulation system, reaching an important step towards personalized medicine that may improve the management of bleeding or thrombosis. Furthermore, POC-TG would be especially beneficial for under-developed communities that suffer the most from the lack of laboratory resources.

In conclusion, the current thesis is a major step forward to a more comprehensive and timely assessment of blood to improve the management of coagulation disorders.

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NEDERLANDS SAMENVATTING

Dit proefschrift richt zich op de innovatie van trombine generatie (TG) assays en de toepassing van TG assays in klinisch en fundamenteel hemostaseonderzoek. **Hoofdstukken 2-4** beschrijven de ontwikkeling en validatie van een nieuwe volbloed TG-test voor het bestuderen van de betrokkenheid van bloedcellen bij stolling, evenals de verkenning van de volbloed-TG-capaciteit van cirrotische patiënten met behulp van een geoptimaliseerde "near-patient testing" volbloed-TG prototype. In **hoofdstuk 5** wordt het momenteel beschikbare bewijs over de toegevoegde waarde van bloedcellen bij TG-testen besproken. In de **hoofdstukken 6 en 7** gebruikten we plasma-TG-assays respectievelijk voor de karakterisering van het stollingsfenotype bij mensen die leven met HIV en onderzochten we het gebruik van TG als een tussenliggend fenotype om nieuwe stollingsgerelateerde genetische varianten te ontdekken.

In **hoofdstuk 2** hebben we een nieuwe fluorogene volbloed TG-test ontwikkeld met een goede reproduceerbaarheid. Deze test is een grote stap voorwaarts vergeleken met eerdere volbloed TG-testen die een slechte reproduceerbaarheid hadden [5], of die vatbaar waren voor contactactivering geïnduceerd door het filterpapier in de test [6]. Het aantal bloedplaatjes en erytrocyten, evenals de (in)activering van bloedplaatjes, bleken cruciale determinanten van volbloed TG te zijn, wat onze hypothese ondersteunt dat onze volbloed-TG-test de invloed van bloedcellen op de bloedstolling omvat. Rode bloedcellen versterken de snelheid van TG, zelfs bij hoge bloedplaatjes aantallen. We bepaalden ook de referentiewaarden van deze test bij een gezonde populatie en bestudeerden deze in relatie tot leeftijd, geslacht, gebruik van orale anticonceptie en het aantal bloedcellen.

In **hoofdstuk 3** hebben we volbloed-TG-profielen van cirrotische patiënten bestudeerd met een geoptimaliseerde "near-patient test" en vonden dat de TG-capaciteit van deze patiënten vergelijkbaar was met die van gezonde controles, wat duidt op een normale bloedstolling, in lijn met het algemeen aanvaarde concept van "rebalanced" hemostase bij deze patiënten [7]. Interessant genoeg was de volbloed-TG-snelheid langzamer bij cirrotische patiënten ondanks een intacte volbloed-TG-capaciteit. Deze waarneming kan worden verklaard door onze eerdere resultaten in gereconstitueerd bloed: het aantal erytrocyten en bloedplaatjes beïnvloedt de TG-snelheid en -capaciteit anders. Een milde afname van het aantal bloedplaatjes en erytrocyten bij cirrose verminderde alleen de snelheid van volbloed-TG, maar niet de totale capaciteit. De balans van het stollingssysteem bij cirrotische patiënten is erg kwetsbaar en kan gemakkelijk kantelen in de richting van een bloedings- of een trombotisch fenotype. Door gebruik te maken van volbloed-TG wordt waarschijnlijk een betere weergave van de situatie in het lichaam verkregen, en volbloed-TG zou de test bij uitstek kunnen zijn om de invloeden van therapie op een fragiel evenwicht, zoals bij levercirrose, te bestuderen en om hun risico op bloedingen of trombose te voorspellen.

In **hoofdstuk 4** vonden we een remmende rol van erytrocyten op de anti stollende functie van geactiveerd proteïne C in volbloed-TG. Het proteïne C-systeem is een belangrijk anticoagulant

systeem en aantasting van dit systeem is een veelvoorkomende oorzaak van trombose [8]. Een verhoogd aantal erytrocyten was gerelateerd aan een verminderd anti-coagulant effect van actief proteïne C (APC) en trombomoduline (TM) in volbloed-TG, zowel in een gezonde populatie als in gereconstitueerde bloedmonsters. Dit effect was niet afhankelijk van bloedplaatjes en was waarschijnlijk gerelateerd aan de fosfolipidensamenstelling van erytrocyten. De remmende rol van erytrocyten op de APC-functie, gecombineerd met de observatie van het versterkende effect van erytrocyten op de volbloed-TG-snelheid, zou een mogelijke verklaring kunnen zijn voor het verhoogde trombotische risico dat verband houdt met een verhoogd aantal erytrocyten, zoals bij polycytemie en erytrocytentransfusies [9, 10].

In **hoofdstuk 5** bespreken we beschikbare continue TG-testen die de betrokkenheid van bloedcellen bij stolling meten, in het bijzonder de fluorogene testen die continue metingen in bloedplaatjesrijk plasma en volbloed mogelijk maken. Ook geven we een overzicht van de invloed van bloedcellen op de bloedstolling, met nadruk op de directe invloed van bloedcellen op TG. Bloedplaatjes versnellen de initiatie en snelheid van TG door blootstelling aan fosfatidylserine, afgifte van granule inhoud en interactie van de oppervlakreceptoren met stollingseiwitten. Erytrocyten zijn ook belangrijke leveranciers van fosfatidylserine en het membranen van rode bloedcellen veroorzaken contactactivering. Bovendien zijn leukocyten en kankercellen belangrijke spelers bij celgemedieerde bloedstolling, omdat ze onder bepaalde omstandigheden weefselfactor tot expressie brengen, pro-coagulante componenten afgeven en plaatjesactivering induceren. We stellen dat het testen van TG in de aanwezigheid van bloedcellen nuttig is om bloedcel gerelateerde stollingsstoornissen te onderscheiden.

In **hoofdstuk 6** hebben we de plasmatische stolbaarheid van HIV-geïnfecteerde individuen met combinatie antiretrovirale therapie onderzocht en vonden dat de plasma TG capaciteit van deze individuen lager was dan die van gezonde controles [10]. Deze waarneming, samen met hun verlaagde protrombinespiegels en verhoogde markers van ontsteking en endotheliale activering, suggereren dat het verhoogde trombotische risico van deze personen niet te wijten was aan hypercoagulabiliteit en hoogstwaarschijnlijk verband hield met verhoogde stimulatie van bloedstolling door endotheliale activering en ontsteking tijdens HIV-infectie. We ontdekten ook dat het gebruik van abacavir geassocieerd was met een protrombotisch TG-profiel in vergelijking met regimes zonder abacavir, ongeacht leeftijd, geslacht en ontsteking, wat nieuwe gegevens opleverde voor het besproken trombotische effect van abacavir [11].

In **hoofdstuk 7** hebben we TG gebruikt als een intermediair stollingsfenotype in een genoomwijde associatiestudie en ontdekten dat het KLKB1-gen gerelateerd was aan de anti-coagulante functie van het proteïne C-systeem. Functionele experimenten toonden aan dat in vitro suppletie van kallikreïne de anti-coagulante functie van TM en APC in TG versterkt. Dit biedt een mogelijk mechanisme voor de eerder waargenomen associatie tussen het KLKB1-gen en trombose [12, 13]. Dit bevestigt ook dat de TM-gemodificeerde TG-test zou kunnen dienen als een hulpmiddel om nieuwe mutaties te ontdekken die verband houden met de proteïne C-route.

Concluderend hebben we in dit proefschrift innovatieve volbloed-TG assays gepresenteerd het meten van de invloed van bloedcellen op TG mogelijk maakt. Hoewel er meer studies over de standaardisatie en klinische relevantie van TG-assays nodig zijn, suggereren onze voorlopige resultaten dat plasma- en volbloed-TG-assays veelbelovende toepassingen hebben in fundamenteel en klinisch onderzoek.

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中文概述

心血管疾病是全球第一大致死疾病。世界卫生组织预计 2017 年全球有 1780 万人死于各种类型的心血管病 [1]。随着全球老龄化的加剧，心血管疾病的威胁也与日俱增。凝血功能紊乱是心血管疾病的主要病因之一。新型凝血功能检测方法的开发有潜力能帮助我们理解凝血系统在心血管疾病中的作用 [2]。

凝血酶生成法（thrombin generation, TG）是一种用于检测血液生成凝血酶潜力的新型方法 [3]。凝血酶是凝血系统中最重要的酶，它既负责切割纤维蛋白原产生纤维网状结构，又能影响血小板功能等其他诸多关键节点。TG 能预估个体的凝血潜能，因而有潜力用于预测出血或血栓的风险。尽管现有的 TG 检测法能对血浆的凝血潜力做比较全面的评估，但是在这些方法中凝血蛋白与血细胞的相互作用无法得到考察 [4].。本论文的主要目的是开发新型的全血凝血酶生成（whole blood [WB] -TG）检测法用以考察血细胞对 TG 的影响。

在**第二章**中，我们开发了一种新型的 WB-TG 荧光检测法，用于连续、高通量地检测全血样品的凝血酶生成潜力 [5]。之前的 WB-TG 检测法受限于较差的可重复性 [6],，或者严重受到滤纸导致的激活通路的干扰 [7].，而本章介绍的全新检测法具有可重复性好，且较少受到接触激活通路的影响。我们还利用该方法考察了血小板和红细胞数目，以及血小板激活剂和抑制剂对 WB-TG 的影响，发现红细胞数目的增高能明显增强凝血酶生成的速率（peak），而血小板的激活程度对凝血酶生成的延迟时间（lag time）有较大影响。我们还收集了 119 名正常供者的全血考察了年龄，性别，口服避孕药以及血细胞数目对 WB-TG 参数的影响，并且建立了正常参考值。

第三章中我们使用了一种本研究所新近开发的小型的 WB-TG 检测原型仪器考察了肝硬化患者的凝血参数 [8]。我们发现肝硬化病人的 WB-TG 总潜能（endogenous thrombin potential until peak, ETPp）与正常人相似，指示正常的凝血潜力，这一发现与最新专家共识的观点一致，即肝硬化患者有一个再平衡的凝血系统 [9]。值得注意的是，肝硬化患者的 WB-TG 速率比正常人低，这可能与这些患者较低的红细胞、血小板数目有关。之前的实验发现当红细胞与血小板的数目达到一定的水平之后，继续增长的细胞数目只影响 TG 速率而不影响 TG 潜力，因此我们推测肝硬化患者略微降低的红细胞、血小板数目导致了 TG 潜力与速率之间的不一致。尽管肝硬化病人获得了一个再平衡的凝血系统，但是这个平衡相比正常人的更加脆弱，意味着这些病人的凝血系统受到外加因素影响时更容易失衡，导致更高的血栓或出血的风险。我们的小型 WB-TG 检测可能对于肝硬化病人凝血功能的监测有一定指导意义，但是它的实际临床价值还有待检验。

在**第四章**中我们发现红细胞会削弱 C 蛋白通路（protein C pathway）的抗凝效果。C 蛋白通路是一个重要的抗凝通路，该通路的异常是一个导致血栓的常见风险因子 [10]。在考察 119 名正常人的 WB-TG 潜力时，我们发现较差的血栓调节蛋白（thrombomodulin）抗凝效果与较高的红细胞数目显著相关，而不与血小板或白细胞数目相关。此外我们还发现血栓调节蛋白和活化的蛋白 C （activated protein C）在 WB-TG 中的抗凝效果都相比富血小板（platelet rich plasma, PRP）-TG 中更弱。我们还制作了重组血液样本，发现随着贫血小板（platelet poor plasma, PPP）中红细胞参杂数目的增加，血栓调节蛋白和活化的蛋白 C 的效果也随之减弱，进一步佐证了红细胞对 C 蛋白通路的削弱效果，且说明该效果不依赖于血小板。这些结果可能部分解释了红血球增多症或者红细胞灌注与增高的血栓风险之间的关联 [11, 12]。

在**第五章**中，我们对现有的基于血细胞的 TG 方法进行了综述 [13]，介绍了 PPP-TG, PRP-TG 和 WB-TG 的发展史，并着重讨论后两种方法了对于研究血细胞相关的凝血疾病的价值。

在**第六章**中我们考察了接受抗逆转录病毒药物联合治疗（combined antiretroviral therapy, cART）的 HIV 感染者的血浆凝血潜能，发现这个人群的 PPP-TG 潜力较正常人更低 [14]。结合 HIV 感染者更低的凝血酶原血浆浓度以及更高的内皮细胞和免疫系统激活标志物水平，我们推测这些之前发现的这些患者增高的血栓风险应该不是由于凝血潜力的变化，而是由于增高的内皮细胞以及免疫系统激活频率。而更频繁的凝血系统激活可能导致了凝血因子的过度消耗，进而解释 HIV 感染者更低的凝血酶原血浆浓度和 TG 潜力。此外我们还发现一种广泛使用的抗逆转录病毒药物阿巴卡韦（abacavir）与增高的 PPP-TG 潜力显著相关，且该关联在校正了年龄、性别和免疫系统激活标志物水平后依然存在。该发现提示需要关注此类药物的致栓风险 [15]。

在**第七章**中我们将 PPP-TG 参数作为中间表型进行了全基因组关联分析（Genome-wide association study，GWAS），考察了与凝血潜力以及 C 蛋白通路抗凝效果相关的基因型。我们发现 *KLKB1* 基因 C 蛋白通路抗凝效果显著相关。此外我们还利用功能性试验验证发现正常血浆中添加纯化的激肽释放酶（kallikrein）能明显提高血栓调节蛋白和活化的蛋白 C 的抗凝效果。值得注意的是，之前的 GWAS 发现 *KLKB1* 基因与血栓风险显著相关 [16, 17]，因此我们的结果可能部分解释了这一关联的机制。

总结与展望

我们开发了新型的 WB-TG 检测方法，并初步考察了 TG 方法在血栓与止血领域的基础和临川研究中的应用潜力。此外，我们的结果显示血细胞特别是红细胞和血小板能明显的影响凝血过程，因此 WB-TG 方法相比传统的血浆检测法可能会提供更为全面的对凝血潜力的评估。

此外，WB-TG 方法不需要对血液进行预处理，因此有潜力基于此开发床旁（point of care, POC）的凝血潜力检测方法。此类的床旁检测方法有望帮助我们更及时精准地检测个体的凝血潜能，有助于精准的预防和治疗。POC-TG 还有望为缺乏复杂实验设备的欠发达地区提供一种更为简易、低廉的解决方案，为此类地区的医疗健康贡献力量。

值得注意的是，目前 WB-TG 仍处于发展的早期阶段。还需要更多的基础研究来解决标准化的难题以及进一步的临床研究来确定其临床价值。

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2019 July, International Society on Thrombosis and Haemostasis (ISTH) annual congress, Melbourne, Australia. ***Wan J***. Pronounced alterations of thrombin generation profiles are found in whole blood compared to plasma of liver disease patients. **Poster presentation**

2019 July, ISTH annual congress, Melbourne, Australia. ***Wan J.*** A novel whole blood thrombin generation model for studying the involvement of blood cells in coagulation. **Oral presentation**

2019 September, 8th International Symposium on Coagulation in Liver Disease, Groningen, the Netherlands. ***Wan J.*** Patients with chronic liver disease have normal thrombin generation capacity in whole blood. **Oral presentation**

AWARD

2021 ISTH congress, Early Career Travel Award

CURRICULUM VITAE

Jun Wan was born on May 30th, 1991 in Yunmeng County of Hubei Province, China. He finished his high school education in Yunmeng and was admitted to Hunan University in Changsha City of Hunan Province in 2009 to study Biotechnology. After obtaining his bachelor's degree in 2013, he was enrolled in the Master program of Biology in the same university. He worked on the selection and applications of cancer-specific DNA aptamers and was awarded the National Scholarship for Graduate students in 2015.

Right after acquiring a master's degree in 2016, he was awarded a scholarship from the China Scholarship Council to fund his PhD training in Maastricht University and Synapse Research Institute, under the supervision of Prof. dr. Tilman Hackeng, Dr. Bas de Laat and Dr. Mark Roest. During his PhD training he worked on the innovation of whole blood thrombin generation assays and applied these assays in both fundamental and clinical studies. He has collaborated with many researchers both within and outside of the Netherlands and has presented his research in several (inter)national conferences. He will work as postdoc in the Lab of prof. dr. Nigel Mackman at the University of North Carolina at Chapel Hill after finishing his PhD.

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