Thrombin generation: innovations and clinical applications

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Contents

Chapter 1

Introduction

Regulation of hemostasis

Activation of blood coagulation is according to Virchow based on the principle that thrombus formation depends on 3 components also known as the triad of Virchow: vessel wall, blood flow and blood composition. These interactions are responsible for preventing excessive blood loss after vascular injury, but also prevent the formation of systemic blood flow occluding thrombi.[1, 2] Vascular injury leads to thrombus formation in which coagulation plays a major role (Figure 1). Two processes that interact synergistically are responsible for blood coagulation: (1) platelet plug formation (also known as primary hemostasis), initiated by exposure of collagen followed by platelet adhesion, activation and aggregation; (2) fibrin formation (also known as secondary hemostasis) starting with tissue factor (TF)-induced activation of the coagulation cascade, resulting in the formation of thrombin that converts fibrinogen into fibrin that stabilizes the formed clot. Another regulatory process that influences the clot formation is fibrinolysis, which is activated when thrombin is formed and causes lysis of the formed clot.[2-5]

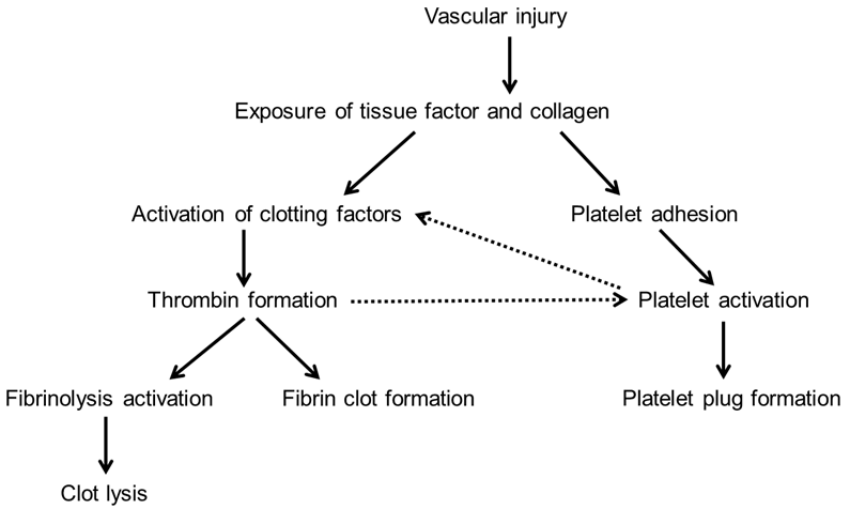

Figure 1: Key steps of thrombus formation. Vascular injury leads to the exposure of collagen and tissue factor. Exposure of collagen initiates primary hemostasis, which results in a platelet plug. Secondary hemostasis, activated by tissue factor, results in the formation of thrombin that converts fibrinogen into fibrin for the stabilization of the formed thrombus. Thrombin formation also leads to activation of fibrinolysis, which mediates lysis of the clot. Adapted from Spahn et al.[5]

The coagulation system can be activated via two separate pathways, known as the intrinsic and extrinsic pathway, that come together in one common pathway (Figure 2). The intrinsic pathway is triggered by contact activation and results in the activation of factor (F)XII and thereby also the activation of FXI and FIX. Physiologically, the extrinsic coagulation pathway is started by tissue factor exposure at the site of a vascular injury. TF binds and activates FVII, resulting in the TF-FVIIa (i.e., the activated form) complex activating FIX and FX. Subsequently, FXa binds and activates cofactor V and, together with prothrombin, they form the prothrombinase complex, which catalyzes the cleavage of prothrombin to thrombin. Thrombin is an important enzyme in the coagulation cascade as it cleaves fibrinogen to fibrin and it evokes the activation of platelets and FV, FVIII and FXI. FVIIIa forms a complex with FIXa and FX, known as the intrinsic tenase complex. The consequent formation of FXa leads to more thrombin formation. Together, these positive feedback loops result in a burst of thrombin and fibrin formation. FXIII is also activated by thrombin and mediates the cross-linking of the fibrin monomers further strengthening the fibrin clot.[2, 6-8]

Platelets are essential for thrombus formation. When platelets are activated, they expose negatively charged phosphatidylserine at the outer leaflet of the activated platelet membrane via an active flip-flop transport mechanism. The phosphatidylserine binds via calcium ions to the γ–carboxyglutamic acid residues in vitamin K dependent coagulation factors. The exposed phosphatidylserine thereby serves as a docking surface for these coagulation factors thereby localizing the clotting process at the site of injury.[2, 6, 7]

Interestingly, thrombin has also anticoagulant effects as it inhibits its own production by forming a complex with endothelium-bound thrombomodulin. Thrombin-bound thrombomodulin activates protein C which in turn inactivates FVa and FVIIIa. Furthermore, thrombin generation is regulated by antithrombins that inhibit FIXa, FXa, FXIa and thrombin. In addition, when the thrombin concentration is high, thrombin activated fibrinolysis inhibitor (TAFI) is produced, which protects the fibrin clot from degradation. Another anticoagulant factor is tissue factor pathway inhibitor (TFPI). This inhibitor forms a complex with FXa and blocks the TF-FVIIa complex, preventing further activation of FXa and FIXa.[2, 6, 7] Since thrombin has multiple roles in the regulation of the coagulation cascade, there is much interest in the continuous measurement of the generation of thrombin during coagulation to detect a hypo- (**bleeding**) or hypercoagulable (**thrombosis**) phenotype.[9, 10]

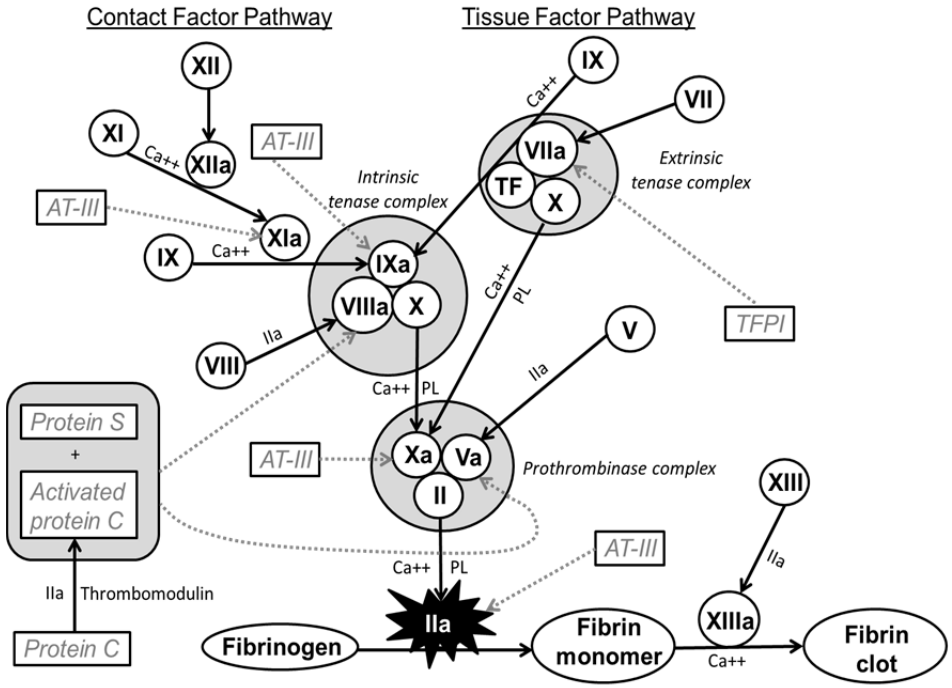

Figure 2: Simplified overview of the coagulation cascade. Contact activation results in the activation of FXI, which in turn activates FIX, while the tissue factor pathway activates FVII and FIX. Both pathways result in the activation of FX that cleaves prothrombin (FII) into thrombin (FIIa). Thrombin converts fibrinogen into fibrin and activates FV, FVIII and FXIII which cross-links the fibrin monomers to form a strong fibrin clot. The dotted lines represent the anticoagulation factors: antithrombin-III (AT-III), protein C and S and tissue factor pathway inhibitor (TFPI).

Bleeding

Excessive bleeding is a frequent event during surgery and after trauma.[11] Fluid resuscitation in bleeding patients is achieved by administration of large volumes of crystalloids and colloids to keep normovolemia and to prevent hypovolemic shock that occurs when 30% of the blood volume is lost.[5, 12] A side effect of the administration of crystalloids and colloids is the dilution of blood plasma and thus coagulation factors, which can lead to the development of dilutional coagulopathy.[3, 11, 12] This coagulopathy is an acquired bleeding disorder, in which coagulation becomes defective due to the loss, consumption and dysfunction of platelets and coagulation factors.[3, 12] It complicates the control of bleeding and is associated with increased morbidity and mortality in surgical and trauma patients. Coagulopathy can also deteriorate into microvascular bleeding, which is difficult to treat because of the hemostatic failure

that develops. In this condition blood is lost from small blood vessels and wounds like the venipuncture site.[12, 13] Life threatening in particular is when coagulopathy develops together with hypothermia and metabolic acidosis, also referred to as the "lethal triad". Each of these three factors aggravates the others, leading to a vicious circle of severe bleeding and even exsanguination. Therefore, timely diagnosis of coagulopathy is essential in order to limit coagulopathy, preferably by the availability of specific near-to-bedside coagulation tests.[5, 14]

Thrombosis

Over the years, the use of anticoagulants to prevent (recurrence of) thrombosis is increasing. The consequence is a need for better monitoring of hemostasis to find the right dose of short- or long-term anticoagulant therapy for each individual patient in order to prevent thrombosis or bleeding.[15, 16] The development of new, referred to as better, anticoagulants contributes to this increasing demand for better monitoring. Unfortunately, with the current coagulation tests available in the hospital, it is not possible to do this for all patients, especially for patients taking the novel oral anti-coagulants (e.g. Rivaroxaban, Dabigatran). This increases the need for the development of new generation hemostatic tests able of monitoring the anticoagulant effects of these new anticoagulants. The ultimate goal is the development of point-of-care devices which can be transferred from bench to bedside or even used at home by the patients for self-monitoring.[16]

Tests available in the clinic

Measuring the hemostatic function of the blood is important in both bleeding and thrombotic disorders.[17] Several techniques are used in the hospital to monitor hemostasis. These techniques vary from clot-based assays to chromogenic based assays, as well as direct chemical measurements and ELISAs.[18] The most commonly used assays are the clot-based assays, that provide a global assessment of the hemostatic function, and chromogenic assays, that can measure the (functional) level of a specific coagulation factor.[18, 19]

Clot-based assays

Clot-based assays are mostly used for monitoring the hemostatic function of bleeding patients with and without heparin administration and for monitoring anticoagulant therapy in patients taking vitamin K antagonists.[19] The principle of these tests can be mechanical (a rotating ball that stops when fibrin is formed), photo-optical (fibrin induced changes in optical density of the sample) or nephelometric (fibrin induced changes in light scattering).[20]

Prothrombin Time (PT). For this assay plasma is used free from platelets and other blood cells.[16, 19] This assay is performed by addition of a reagent containing calcium, phospholipids and TF (derived from different sources like brain, lung, placenta or recombinant technology) to plasma followed by recording the time needed to start fibrin formation. In case of a very low fibrinogen level, the PT cannot be assessed. The PT varies depending on the reagent and coagulometer and ranges between the 10 and 14 seconds.[19, 21] It is prolonged by deficiencies in factors VII, X, V, II, fibrinogen or antibodies against one of these factors. Prolongation of the PT can also occur due to the presence of heparin, fibrin degradation products and inhibitors of fibrinogen cleavage into fibrin. Since there is an excessive amount of phospholipids present in the reagent, it is not possible to detect non-specific inhibitors against anionic phospholipids (e.g. lupus anticoagulants).[19, 22] The most common use of the PT is to monitor patients taking vitamin K-antagonists.[23] To correct for differences in reagents and especially differences in TF, the **international normalized ration** (INR) is used. The INR is achieved by the following formula: INR =[(patient PT)/(mean normal PT)]^{ISI}. In this formula ^{ISI} stands for the sensitivity index of the working thromboplastin relatively to the WHO international standard. For the normal PT it is advised to take the mean of the PT of at least 20 healthy donors and this has to be repeated with every new batch of TF reagent. Although the INR contributes to standardized anticoagulant monitoring, the problems due to variation in combination of the reagent and coagulometer still persist.[19, 23, 24]

Activated Partial Thromboplastin Time (aPTT). Plasma (free from all blood cells) is incubated in this assay with a reagent containing a low phospholipid concentration (e.g. cephalin) and a surface activator (e.g. kaolin, celite, ellagic acid or silica). The incubation is needed to activate optimally factor XII, XI, prekallikrein and high molecular-weight kininogen. Afterwards, calcium is added to the sample and the

clotting time is measured.[19, 21] The aPTT is very dependent of the reagent used and ranges from 20 seconds up to 40 seconds. It is prolonged by deficiencies in factor II, V, VIII, IX, X or fibrinogen. (Non-) specific inhibitors of these factors, fibrin degradation products, lupus anticoagulants and anticoagulants (e.g. heparin, direct thrombin inhibitors, warfarin) also result in the prolongation of the aPTT. Compared to the PT, the aPTT is less sensitive to warfarin and for this reason the PT is used to monitor warfarin treatment.[19, 25, 26] In addition, the aPTT is not optimal for monitoring many other anticoagulants. For example, it is insensitive to low-molecular-weight heparins (LMWH), too sensitive to hirudin treatment and inaccurate in monitoring the combination of heparin together with abciximab (an inhibitor of glycoprotein (GP) IIbIIIa). Problems with the aPTT can occur when the interval between blood collection and aPTT testing increases. Platelet fragmentation occurs in time, which will contribute to the aPTT assay due to the low phospholipid concentration present in the reagent, leading to misleading results.[16]

Activated Clotting Time (ACT). For this assay, whole blood is put into a coagulation activator (e.g. glass, kaolin, celite) and the time needed to form the clot is measured. Normally the ACT ranges from 70 up to 180 seconds depending of the activator used. The ACT is frequently used for monitoring treatment with high concentrations of heparin or bivalirudin, which can not be measured with the aPTT test.[19, 22] Depending on the intervention, different ranges of the anticoagulant are needed and these can be adjusted using the ACT.[19] The ACT does not correlate well with other coagulation tests and, like the aPTT, it is insensitive to LMWH and too sensitive to hirudin.[16, 19]

Thrombin Clotting Time (TCT). This assay is performed by addition of an excessive amount of thrombin to cell-free plasma. It is prolonged in patients with a low fibrinogen level, with dysfibrinogenemia (qualitative fibrinogen problem) or with elevated fibrinogen degradation products (often seen in patients with disseminated intravascular coagulation). The TCT is also prolonged in plasma samples containing heparin and other direct thrombin inhibitors.[19, 21, 22]

Ecarin Clotting Time (ECT). In this test, cell-free plasma is incubated with a snake venom (*Echis carinatus*) that converts prothrombin into meizothrombin. Meizothrombin is an intermediate form of prothrombin that is sensitive to the inhibition of direct thrombin inhibitors, making this assay useful for monitoring

treatment with these inhibitors. The ECT still has to be standardized and is not sensitive to all coagulation disorders and heparin treatment. A chromogenic variant of this assay is also available.[19, 27]

Chromogenic assays

The most commonly used chromogenic assays are anti-FXa assays to measure the concentration of heparin and LMWH. These assays use a chromogenic substrate that can be cleaved by FXa.[19] The most commonly used chromophore is *para-nitroaniline*.[20] When FXa cleaves the substrate, the chromophore linked on the substrate is released and emits a color that can be detected with a spectrophotometer. The amount of FXa is proportional to the emitted color. When a known amount of FXa is added to plasma containing an unknown amount of heparin, heparin will induce FXa inhibition by antithrombin, leaving less FXa available for substrate cleavage. From a calibration curve prepared with a pooled normal plasma added with known and increasing heparin concentrations, it is possible to calculate the heparin concentration in the sample.[19]

Factor assays

These assays are often based on an immunological principle. Specific antibodies can be attached to latex microparticles and when the antigen binds to the antibody agglutination of many particles will occur. Monochromatic light is used in these assays that can be absorbed by the microparticles when their size approaches the wavelength of the light. The amount of light absorption is proportional to the amount of antigen-antibody agglutination present and therefore also to the amount of antigen present.[20]

Clauss method

This assay is used to measure the fibrinogen concentration. Excessive amounts of thrombin are added to a sample and the time is measured until the clot is formed. The concentration of fibrinogen in a sample is than calculated from a calibration curve. Hemodilution with colloids can interfere with this assay leading to falsely high fibrinogen concentrations.[28]

Platelet function

Platelet function tests are used for diagnosing platelet function disorders and monitoring anti-platelet therapy.[29] This monitoring is suitable for primary or secondary prevention of cardiovascular disease and the aim is to decrease the incidence of acute cerebro- and cardiovascular events. The therapy works primarily by decreasing platelet aggregation, but also by acting as anti-coagulant: activated platelets facilitate thrombin generation (TG) by providing a catalytic cell surface based on phosphatidyl serine exposure on which coagulation reactions may occur releasing activated FV. On the contrary, anticoagulants may also alter platelet function.[28, 30, 31] Testing the platelet function is especially critical in the perioperative settings since they play a role in overall hemostasis.[29, 32] Turbidimetric platelet aggregometry is the clinical standard of platelet function testing. The disadvantages of this technique are that it is labor-intensive, costly, time-consuming, needs high degree of experience and expertise to perform and interpret.[28, 29]

Thromboelastography

Thromboelastography is a method to assess the overall hemostatic function of a blood sample by measuring the viscoelastic changes in time under almost static conditions.[28] This assay is able to monitor the process starting from the earliest point of fibrin formation, up to clot stability, clot retraction and clot lysis.[28, 33, 34] There are different reagents to test the fibrin formation in different conditions: samples can be activated intrinsically and extrinsically, with and without the contribution of platelets (by addition of cytochalasin D, a platelet inhibitor), with a heparinase to exclude the contributing effect of heparin, and with aprotinin, a fibrinolysis inhibitor to investigate the fybrinolytic pathway.[28] It can be used for hemostatic monitoring during liver and cardiothoracic surgery, massive hemorrhage, hypo- and hypercoagulable states, hyperfibrinolysis, but also for monitoring pharmacological treatment with anti- and procoagulant agents.[28, 35] A major advantage is that results are received within minutes, in contrast to other routine coagulation tests which are performed with plasma and therefore need centrifugation steps.[36] Disadvantages are that a normal thromboelastograph curve does not exclude a defect in the haemostatic process (e.g. factor deficiency, vitamin K antagonist treatment, …) as the method is highly dependent on fibrinogen content and less on other coagulation factors.[34] There are some problems related to standardization of the method, but it already provides an inexpensive snapshot of a part of the hemostasis at the bedside of the patient. The

increasing use of thromboelastography illustrates the clinical need for POC assays to diagnose coagulopathies and to guide the management of patients with coagulation disturbances.[33, 37]

Sonoclot

The Sonoclot measures the viscoelastic changes of blood or plasma in time, as well as the fibrin formation kinetics, the clot development and platelet function. Differently than thromboelastography, it is more sensitive to the viscosity, especially to the initial part of the fibrin formation.[28, 38] The disadvantages are that this assay has a poor reproducibility and it is influenced by gender, age and platelet count.[28, 39]

Point-of-care devices available in the clinic

The most commonly used POC device in clinic is the ACT. Other POC assays available are those to measure INR, anti-Xa, ECT and thromboelastography. Nowadays, self-testing of the INR measurement by the patient at home is gaining more interest. Studies have shown that self-management using an INR POC device is safe for selected patients and did not reduce the quality of care compared to INR testing provided by specialized anticoagulant clinics.[19] Unfortunately, the problem with POC devices is that they are difficult to standardize. For example, blood collection site, processing of the sample and age all play a role.[19, 28] Furthermore, difference in laboratory equipment, activators and reagents, will alter the assay results. POC devices should be adequately maintained, supervised and quality controls have to be done on a regular basis. Non-laboratory persons are running these tests which might influence the test-results.[19, 28] Since POC testing is often more expensive than centralized assays performed in the hospital, cost-effectiveness studies have to be done to justify the widespread use of these tests.[19] For some POC devices this was already done and it was shown that self-monitoring not only resulted in a better control of the anticoagulation, but also reduced the overall costs.[16, 40-42]

Figure 3: Prof. Hemker and Prof. Béguin performing TG with the subsampling method, the precursor of the current CAT assay (Paris, 1985). His law about thrombosis and hemostasis is: "The more thrombin the more thrombosis but the less bleeding, the less thrombin the less thrombosis but the more bleeding".[48]

The Calibrated Automated Thrombogram assay

The Calibrated Automated Thrombogram (CAT) assay developed by Prof. Hemker and co-workers (Figure 3), is a versatile tool in the field of thrombosis and hemostasis. This assay makes it possible to distinguish normal conditions from hypo- and hypercoagulable states.[43-45] The CAT assay was designed to measure TG in platelet rich (PRP) and poor plasma (PPP).[43] Small adaptions to this assay, made it possible to investigate specific parts of the coagulation cascade, e.g. activating samples with FIXa instead of tissue factor enables accurate measurement of FVIII levels in hemophiliac A patients.[46] A typical TG curve is shown in Figure 4. The most important parameters are the lagtime, the endogenous thrombin potential (ETP, area under the curve), the peak height and the velocity index. The lagtime is the time needed to produce the first traces of thrombin and has a good correlation with clotting time assays like the PT. The ETP stands for the total amount of active thrombin formed during the whole experiment. The peak height is the maximal active thrombin formed and the velocity index is a measure for the velocity of active thrombin formation.[43, 47]

Until recently it was not possible to measure TG in whole blood. A reliable TG assay in whole blood is important because it is closer to physiology compared to current

practice. Besides this conceptual advantage there is the practical advantage to disregard centrifugation of the blood sample, thereby reducing time and errors that can occur. A more technical advantage is that whole blood is ready to use, which gives the opportunity to measure TG in a sample directly without addition of an anticoagulant. This opens the way to point-of-care TG testing via only a finger prick.[43]
The work done by Prof. Dr. Rafael Apitz was crucial for the development of the whole blood TG assay.[48] Essentially two problems had to be resolved to measure TG in whole blood with the regular CAT assay. First of all, a thin layer had to be created with a porous matrix to overcome the problems related to the presence of red blood cells such as red blood cell sedimentation, clustering and retraction leading to highly erratic signals. Secondly, the substrate used in the regular CAT assay is quenched by hemoglobin, therefore another thrombin substrate (rhodamine-based) had to be used for performing TG in whole blood. The whole blood CAT assay is currently validated and proved to have a good reproducibility and coefficient of variation.[43] Moreover, it was also demonstrated that this assay can be used for TG in whole blood of mice.[49] Since it is not fully automated, up to now it is only possible to use it in research settings. Standardization and other modifications should be applied before it can be introduced into the clinic for the diagnosis and treatment of patients.[43]
Clotting time assays that are currently being used in the clinic, e.g. the PT and the aPTT, are not able to detect hypercoagulability in blood samples and only indicate the time that a sample needs to produce 1% of its thrombin capacity, leaving the other 99% undetected. Moreover, it is impossible to detect the contribution of the natural anticoagulants (e.g. antithrombin, protein C and S) with these clotting time assays.[50, 51] The CAT assay is very useful, as it is capable to detect hypercoagulability and the contribution of natural anticoagulants. Except fibrin formation and clot firmness (which can be achieved by thromboelastography measurements), the CAT assay is able of giving an overall function of the coagulation status.[43, 51, 52]

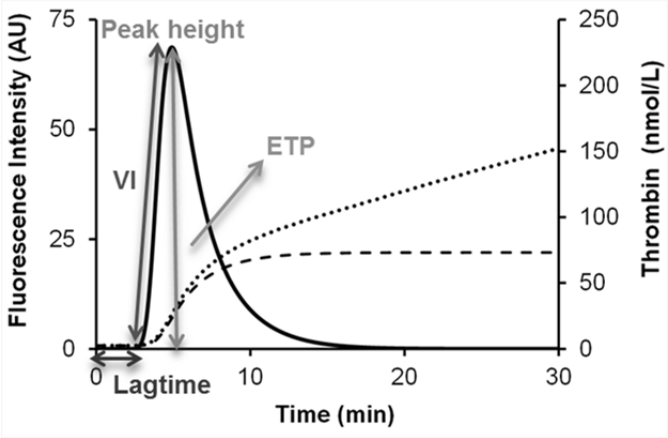

Figure 4: Representative TG curve with the most important parameters: peak height, ETP (endogenous thrombin potential, area under the curve), lagtime, VI (velocity index).

Outline of this thesis

This thesis describes the future generation of TG assays including different applications in whole blood and plasma. In **Chapter 2** the technical validation of a new method is described to measure TG in whole blood based on the CAT method, called the "WB-CAT assay". It is demonstrated to have a good reproducibility and coefficient of variation and was able to detect changes in coagulation related to both treatment and disease. In addition to human blood, the WB-CAT assay using mice blood is further discussed in **Chapter 3**. In short, we describe the technical validation of the WB-CAT assay in mouse blood followed by showing a proof-of-principle study in Bmal1-KO mice. These mice have a prothrombotic phenotype, but display contrasting results in different types of clotting assays. WB-TG in these mice appeared to be increased compared to their WT littermates, indicating that the WB-CAT assay correlated well with their prothrombotic phenotype. In **Chapter 4** we investigated the effect of hypoxia on TG due to an increase in altitude. For this study we went to the Mont Blanc massive with 30 healthy donors and investigated TG using different methods including whole blood TG and the classic plasma CAT method. As physical activity can influence hemostasis by increasing FVIII and VWF levels, irrespectively of oxygen levels, we compared two different groups; an active climbing group and an inactive cable car group. In **Chapter 5** it is shown that by the use of a different activator (FIXa) it is

possible to measure FVIII levels accurately under 2%. The assay was developed to investigate whether the FVIII level in severe hemophiliac patients correlates with the difference in bleeding phenotype in these patients. Another example in which TG plays a role in the diagnosis of a hematological disease is described in **Chapter 6**. In this chapter we investigated whether TG has a role in the diagnosis of the antiphospholipid syndrome. β2-glycoprotein I is known as the major antigen in this syndrome and we studied the effect of different β2-glycoprotein I-conformations on TG and included a short review summary. **Chapter 7** is focusing on the effect of blood/plasma dilution on TG and thromboelastography measurements. First the effect of *in vitro* dilution was studied with and without the addition of various transfusion products (factor concentrates and blood cells). Secondly, the effect of *in vivo* dilution due to the cardiopulmonary bypass procedure was also investigated. **Chapter 8** describes the effect of transfusion of fresh frozen plasma and fibrinogen concentrates to severe bleeding patients. TG and thromboelastography measurements were done on samples taken before and after transfusion. The General Discussion of this thesis is described in **Chapter 9**.

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Chapter 2

Whole Blood Thrombin Generation Monitored with a Calibrated Automated Thrombogram-Based Assay

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Abstract

***Background*:** The calibrated automated thrombogram (CAT) assay in plasma is a versatile tool to investigate patients with hypo- or hypercoagulable phenotypes. The objective was to make this method applicable for whole blood measurements.

***Methods*:** Thin-layer technology and the use of a rhodamine 110–based thrombin substrate appear to be essential for a reliable thrombin generation (TG) assay in whole blood. Using this knowledge we developed a whole blood CAT-based assay (WB-CAT).

***Results*:** We demonstrated that the WB-CAT is a sensitive and rapid screening test to assess functioning of the hemostatic system a step closer to physiology than the TG assay in plasma. Under conditions of low tissue factor concentration (0.5 pmol/L) and 50 vol% blood, the intra-assay coefficient of variation (CV) of the thrombogram parameters, endogenous thrombin potential (ETP) and thrombin peak height (TP), were 6.7% and 6.5%, respectively. The respective inter-assay CVs were 12% and 11%. The mean inter-individual variation (SD) of forty healthy volunteers was 633 (146) nmol•min/L for the ETP and 128 (23) nmol/L for the thrombin peak. Surprisingly, erythrocytes contributed more than platelets to the procoagulant blood cell membranes for optimal TG. Statistically significant ($P < 0.001$) and potentially clinically significant correlations were observed between circulating factor VIII levels in blood of hemophilia A patients and ETP ($r = 0.62$) and TP ($r = 0.58$).

***Conclusion**:* We have developed a reliable method to measure TG in whole blood. The assay can be performed with a drop of blood and may provide a useful measurement of TG under more physiological conditions than plasma.

Introduction

The thrombin generation (TG) assay is increasingly being recognized as a versatile diagnostic tool in the field of thrombosis and hemostasis. It is well accepted that the transient TG profile in clotting plasma is a better determinant of the overall function of the hemostatic system than clotting-time based assays are, e.g. prothrombin time (PT) and activated Partial Thromboplastin Time (aPTT).[1-5] TG in plasma is most accurately measured with the calibrated automated thrombogram (CAT) assay as developed by Hemker and co-workers.[6-9] This method has been designed for application with platelet-free and platelet-rich plasma as analytes. The method is, however, not applicable for measuring TG in whole blood. Variable quenching of the fluorescent signal by hemoglobin and red blood cells that sediment, cluster and retract with the clot, lead to highly erratic signals.[10, 11] The use of fibrin as an indicator product for TG in whole blood, as in thromboelastography (TEG), does not reflect the thrombin-generating capacity of patients because fibrinogen as a substrate is quickly exhausted and therefore informs about TG only at earliest phases of the coagulation process.[5, 12] The information about clot mechanical properties that is obtained in later stages of a TEG experiment is related to TG in a complicated and nonunequivocal manner. Mathematical treatment of the TEG signal so as to make it resemble to a TG curve therefore does not contribute except to confusion.[13, 14]

A reliable whole blood thrombin generation assay is of importance because it enables us to go one step closer to physiological conditions than is possible in present practice. Apart from this conceptual advantage there is the practical advantage that no centrifugation step is required, which opens the way to point-of-care TG measurements. We found that the problems caused by the presence of red blood cells can be overcome by dispersing the blood in a porous matrix, thereby creating a thin layer of blood and by using rhodamine-110 as a signalling product.[15] By these modifications of the plasma CAT assay, we acceptably reduced quenching and issues related to unequal distribution of red blood cells. We here report the technical validation of a whole blood CAT-based assay (WB-CAT) of acceptable imprecision that measures TG in a thin layer of whole blood. As proof of concept, we compared the whole blood thrombogram parameters with factor VIII plasma concentrations in hemophilia A patients.

Materials and Methods

Reagents

BSA5 buffer contains 20 mmol/L HEPES, 140 mmol/L NaCl and 5 mg/mL bovine serum albumin (BSA; Sigma) with a pH of 7.35. The rhodamine-based thrombin substrate (P_2Rho) was from Diagnostica Stago. The calibrator, $\alpha_2$macroglobulin-thrombin (α2M-T) complex, was prepared in-house as previously described.[7] Recombinant human tissue factor (TF; Innovin®) was from Siemens Healthcare Diagnostics Inc. The FXIIa inhibitor corn trypsin inhibitor (CTI) was purchased from Haematologic Technologies, Inc. Phospholipid vesicles (PV) contained 20 mol% phosphatidylserine, 60 mol% phosphatidylcholine and 20 mol% phosphatidyl-ethanolamine (Avanti).

Study Population

Blood was taken from healthy adult volunteers who gave full informed consent according to the Helsinki declaration and had not taken any medication that interferes with hemostasis. Twenty-seven adult hemophilia A patients with a mean (SD) age of 31 (10) years old routinely treated in the Lyon Clinical Hemostasis Unit (France) were included in the study after the patients provided written informed consent. The study met all institutional ethics requirements. Of these twenty-seven hemophilia A patients, 7 were severe (factor VIII:C <1 IU/dL), 9 moderate (factor VIII = 1 – 5 IU/dL) and 11 mild hemophiliacs (factor VIII = 5 - 30 IU/dL).

Blood and plasma preparation

Blood (9 volumes) was aseptically drawn in vacutainer tubes (BD Vacutainer System) containing 3.2% sodium citrate (1 volume), from the antecubital vein of healthy study participants and hemophilia patients. The blood was kept at room temperature (± 22°C) and used within 4 hours from withdrawal. Platelet rich plasma (PRP) was obtained by centrifugation of blood at 240*g* for 15 minutes and platelet poor plasma (PPP) by centrifugation twice at 2630*g* for 10 min.

Preparation of reconstituted blood

Red blood cells were isolated by centrifugation of the blood at 240*g* for 15 min to obtain PRP. The PRP was discarded and the red blood cell suspension was resuspended in a HEPES buffer (10 mmol/L HEPES, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L

$MgCl_2$, 0.1 weight% glucose and 0.1 weight% BSA, pH 7.45). This procedure was repeated three times to assure that virtually all platelets and leukocytes were removed from the red blood cell suspension. Samples of this suspension were diluted with HEPES buffer up to different red blood cell concentrations. One volume of diluted red blood cell suspension was mixed with one volume autologous PPP to obtain samples with a different hematocrit level, but with a constant amount of coagulation factors. The blood cell count and hematocrit were determined with a thrombocounter (Beckman Coulter).

Thrombin generation measurement in whole blood.

Paper discs of 5 mm in diameter and 180 µm thickness (Whatman 589/1, Whatman GmbH) were placed in flat bottom wells of a 96-wells polystyrene microplate (Thermo Electron Corporation). Separately, 30 µL of citrated whole blood was mixed with 10 µL P_2Rho (1.8 mmol/L) and 20 µL TF and $CaCl_2$ (1.5 pmol/L and 50 mmol/L, respectively). For TG in plasma, 30 µL PPP was mixed with 10 µL P_2Rho (1.8 mmol/L) and 20 µL trigger solution containing TF (3 pmol/L), PV (12 µmol/L) and $CaCl_2$ (50 mmol/L). Immediately after mixing, a sample of 5 µL was pipetted on paper disks and covered with 40 µL of mineral oil (USB Corporation). The final concentrations for whole blood were: 50% whole blood, 0.5 pmol/L TF, 16.7 mmol/L $CaCl_2$, and 300 µmol/L P_2Rho; and for PPP: 50% PPP, 1 pmol/L TF, 4 µmol/L PV, 16.7 mmol/L $CaCl_2$, and 300 µmol/L P_2Rho. In a parallel calibration experiment the TF containing solution was replaced by 20 µL human thrombin calibrator (α2M-T, 300 nmol/L thrombin activity). Fluorescence signal was recorded with a Fluoroskan Ascent microplate fluorometer with λ_{ex} = 485 nm and λ_{em} = 538 nm (Thermolabsystems). Samples were run at least in triplicate and the calibrated TG curves were calculated as previously described.[16] All procedures were performed at 37°C.

Plasma FVIII:C

FVIII activity was measured using a deficient plasma kit, FVIII deficient plasma (Precision BioLogic) on a Destiny Max Haemostasis analyzer (T-Coag).

Statistical analysis

Statistical analyses were performed using Graph Pad Instat 3.0 software. The Spearman statistic was applied for correlation analyses. A *P* value of <0.05 was considered statistically significant. The thrombograms shown are the mean of a set of

three separate measurements. The thrombin generation parameters were calculated from the separate curves and shown as the mean ± SD.

Results

Reaction profiles

Despite the presence of red blood cells, the stability of the fluorescence signal in blood was as good as that of the signal in plasma. The average variation of the signal during the final slope, i.e. the in situ residual activity of the α2-macroglobulin-thrombin (α_2M-T) complex formed during the assay in plasma and blood was respectively 0.1% and 0.4%. Fluorescence tracings, showing the response of the WB-CAT assay to activated whole blood, had qualitatively the same profiles as the profiles obtained with PPP derived from the same blood sample (Figure 1A). However, it can be seen that despite nearly identical reaction conditions, the increase of the fluorescence signal was much stronger in plasma compared with whole blood. The higher signals in the plasma experiments can be explained by the absence of the fluorescence scattering effects of the red blood cells and the quenching effect of hemoglobin. Collectively, these results demonstrate that the TG assay can be performed in a thin porous matrix and that such matrices minimize red blood cell induced scattering of the fluorescence readings. However, calibration of the whole blood TG assay remains an important issue because of varying hemolysis and/or hematocrit of the blood samples.

A representative fluorescence tracing (mean of three replicates and solid lines representing SD values) measured with the WB-CAT assay responding to the reaction between P_2Rho (300 µmol/L) and α2M-T (100 nmol/L thrombin activity) in citrated blood is shown in Figure 1B. To examine whether the fluorescence intensity changes in time deviates from linearity, the first derivative of the mean of three replicates was plotted versus time (dotted line). We conclude from these findings that a correction for substrate exhaustion and inner filter effect, as observed in the classical CAT method using plasma as an analyte, is not required for TG measurements in a thin layer of whole blood. The linear slope of the fluorescence intensity vs time curve yields the calibration factor that converts the fluorescent signal of the raw TG curve into a molar thrombin concentration.

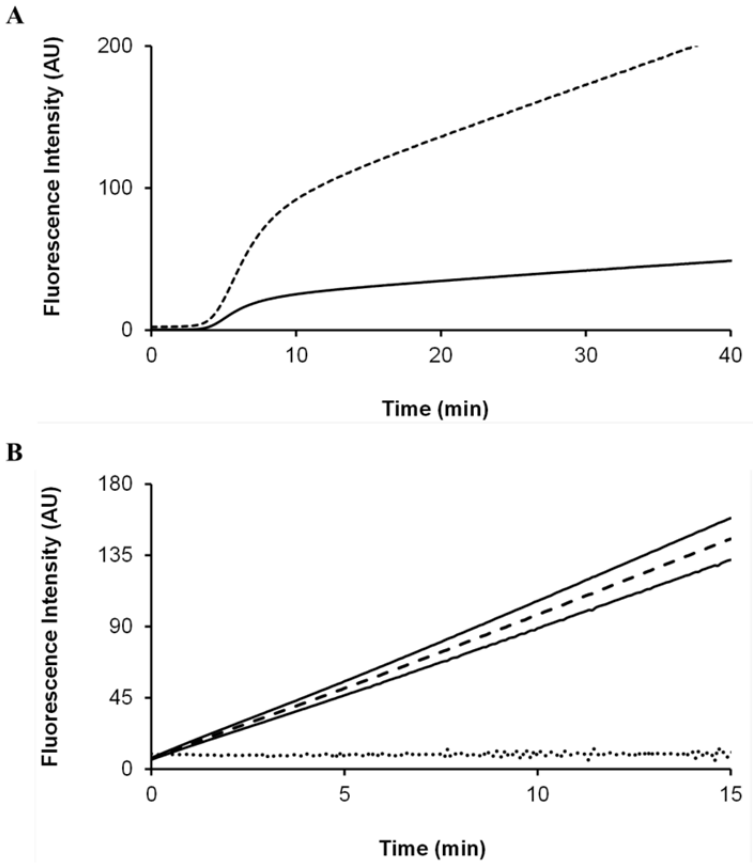

Figure 1. Fluorescence tracings showing the response of the WB-CAT system to TG in activated PPP and whole blood. A) Citrated PPP was activated with 0.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ and 4 µmol/L phospholipids in the presence of 300 µmol/L P_2Rho (dashed line) and citrated whole blood was activated with 0.5 pmol/L TF, 16.7 mmol/L $CaCl_2$ in the presence of 300 µmol/L P_2Rho (solid line). B) Response of the WB-CAT system (dashed line; mean of 3 replicates) to the reaction between P_2Rho (300 µmol/L) and α2M-T (100 nmol/L thrombin activity) in citrated blood. The solid lines present the SD values of the 3 replicates. The first derivative of the calibrator response is presented by the dotted line.

Conversion of raw data into thrombin concentration

To obtain TG profiles, a model was fitted (Figure 2, dotted line) to the raw fluorescence data as previously described.[16] The goodness of fit was apparent from the small residuals (< 1% of the raw values) that were randomly scattered around zero (data not shown). The increase in fluorescence intensity due to formation of α2M-T activity was subtracted from the total fluorescence intensity (Figure 2, dashed line). The first derivative of this corrected fluorescence-time curve and conversion of fluorescence

intensity with the calibration factor yielded a thrombogram with thrombin activity expressed as nmol/L (Figure 2, solid line). To describe the thrombogram, the following parameters were calculated: endogenous thrombin potential (ETP, expressed as area under the curve; nmol·min/L), lagtime (LT; min), thrombin peak (TP; nmol/L), and time-to-thrombin peak (TTP; min).

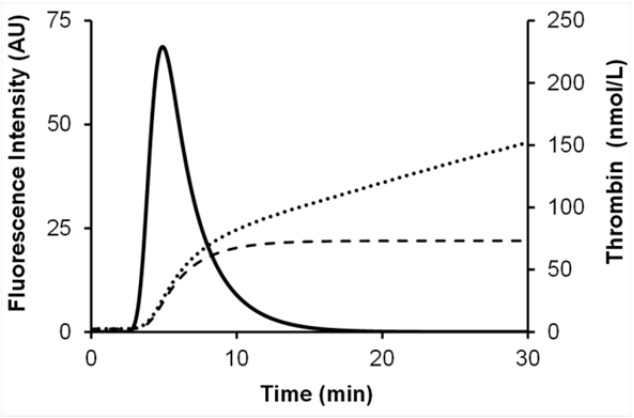

Figure 2. Typical thrombogram. Thrombin generation in whole blood was induced by adding TF (0.5 pmol/L) and $CaCl_2$ (16.7 mmol/L) in the presence of 300 µmol/L P_2Rho. A model was fitted against the raw data (dotted line) as previously described.[16] The goodness of fit was apparent from small residues (< 1% of the raw values) that randomly scattered around zero. Raw thrombin generation data (dotted line) were corrected for residual α2M-T activity (dashed line). The solid line shows the corrected and calibrated thrombin generation curve in nmol/L. AU, arbitrary units.

Optimal TF concentration for measuring TG in whole blood

We measured TG at varying TF concentrations in the presence of P_2Rho (300 µmol/L) and $CaCl_2$ (16.7 mmol/L). The thrombogram parameters as a function of the TF concentration are depicted in Table 1, in which the table values are given as the mean ± SD of three measurements. Maximal thrombin generation was observed with TF concentrations higher than 2 pmol/L. We recommend performing the assay with a low (0.5 pmol/L) TF concentration to include also the contribution of the intrinsic pathway factors VIII, IX and XI. We note that in the absence of exogenous TF, a substantial amount of thrombin was produced. To investigate whether this TG is the result of pre-analytical contact activation, the assay was carried out with blood collected on citrate and in the presence of a FXIIa inhibitor (i.e. 50 µg/mL CTI). A comparison of the thrombogram parameters revealed that when blood is collected on CTI, ETP decreased slightly (8%), the thrombin peak decreased by 52% and the lagtime was prolonged 4-

fold. Because of the artifactual and variable character of contact activation, collection of blood on citrate with an inhibitor of FXIIa is recommended when less than 1 pmol/L TF is used to trigger blood coagulation.

Table 1. Recombinant tissue factor (TF) dependency of the WB-CAT parameters.*

TF (pmol/L)	ETP (nmol.min/L)	LT (min)	TP(nmol/L)	TTP(min)
0	678 ± 29	8.1 ± 1.2	87 ± 5	12.9 ± 0.6
0.5	627 ± 14	4.8 ± 0.1	148 ± 5	6.9 ± 0.1
1.0	710 ± 53	4.2 ± 0.2	168 ± 19	6.3 ± 0.2
2.0	691 ± 118	3.3 ± 0.1	176 ± 10	5.1 ± 0.3
5.0	701 ± 15	2.2 ± 0.1	177 ± 9	4.2 ± 0.1

*Data are means ± SD (N=3). ETP, endogenous thrombin potential expressed as area under the curve, LT, lagtime, TP, thrombin peak, and TTP, time-to-thrombin peak

Source of whole blood procoagulant membranes

Thrombin generation in TF-activated plasma requires the presence of procoagulant phospholipid membranes for the assembly of thrombin generating enzyme complexes.[17] It has been shown that platelets are an essential contributor to TG in PRP that is triggered with a low amount of TF.[18] However, it has also been suggested that red blood cell membranes may contribute to TG.[19, 20] With the tool described in the present paper, this issue can now be studied in detail.

The data shown in Figure 3A illustrate that addition of synthetic PV (10 µmol/L) to whole blood hardly influences TG, whereas such addition is essential for TG in PPP. This finding demonstrates that blood cells provide sufficient procoagulant phospholipids for optimal TG. The contribution of red blood cells was demonstrated by a partial restoration of TG when 1 volume of PPP was mixed with 1 volume of washed red blood cell to obtain a hematocrit of 38% (Figure 3B). A full restoration was not expected because the plasma fraction was diluted as well, resulting in sub-optimal coagulation factor levels.

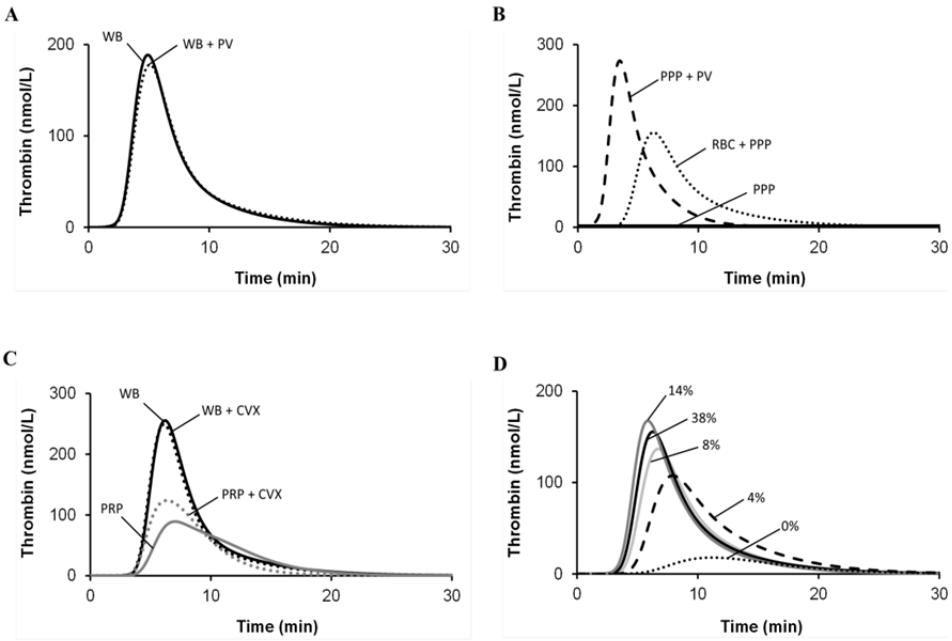

Figure 3: Phospholipid dependency. A) Whole blood was incubated with P_2Rho (300 µmol/L), $CaCl_2$ (16.7 mmol/L) and TF (0.5 pmol/L) in the absence (solid line) and presence (dotted line) of phospholipid vesicles (PV). B) TG measurement in reconstituted blood composed of 1 volume of washed red blood cells and 1 volume of platelet poor plasma (PPP) with a final hematocrit of 38% (dotted line), PPP (solid, flat line) and PPP containing 10 µmol/L PV (dashed line). C) PRP and whole blood TG measurements in the absence (solid line) and presence (dotted line) of convulxin (50 ng/ml). D) TG in mixtures that contained varying numbers of red blood cells and a fixed amount of PPP up to the indicated hematocrit levels.

In order to discriminate between platelets and red blood cells, convulxin, a potent platelet activator that acts via glycoprotein VI,[21] was added to whole blood and to PRP. Whereas in PRP convulxin had a significant stimulating effect, there was no effect in whole blood (Figure 3C). Demonstration of the dependency of the TG on hematocrit provides evidence that red blood cells are the major source of procoagulant membranes. When TG was measured in mixtures that contained varying amounts of washed red blood cells, devoid of platelets and leukocytes, and a fixed amount of PPP, the TP values increased with hematocrit and reached a plateau value at about 14% (Figure 3D). Addition of 10 µmol/L phospholipid vesicles to the reconstituted blood samples normalized the thrombin peak heights in samples with a hematocrit of less than 14% (data not shown).

Precision of the WB-CAT assay

To obtain intra-assay precision data, we performed TG measurements under similar conditions (0.5 pmol/L TF, 300 µmol/L P_2Rho and 16.7 mmol/L $CaCl_2$) with the WB-CAT method (15 replicates). The mean values and the coefficient of variation (CV) of the thrombogram parameters are depicted in Table 2. Inter-assay precision of the thrombogram parameters were calculated from 14 independent measurements in triplicate of a single whole blood sample. Inter-assay variation could not be assessed from measurements over a time-period longer than one day because of donor differences and changes that occurred during storage of the blood sample. The mean values and the inter-assay CV are depicted in Table 2. The results indicate that the intra-assay and inter-assay precision are sufficient for useful application of the assay in clinical and fundamental research.

Table 2. Intra- and inter-assay precision of the WB-CAT parameters.*

	Intra-assay variation (N=15)		**Inter-assay variation (N=14)**	
Parameter	**Mean ± SD**	**CV (%)**	**Mean ± SD**	**CV (%)**
ETP (nmol.min/L)	907 ± 60	6.7	633 ± 74	11.7
LT (min)	3.3 ± 0.1	3.7	4.3 ± 0.4	9.7
TP (nmol/L)	175 ± 11	6.5	131 ± 14	10.7
TTP (min)	5.8 ± 0.1	2.0	6.6 ± 0.4	6.2

*ETP, endogenous thrombin potential expressed as area under the curve, LT, lagtime, TP, thrombin peak, and TTP, time-to-thrombin peak

Inter-individual variation

To find the variation of thrombogram parameters in healthy individuals, WB-CAT assays were performed with blood from 40 volunteers. The mean values ± SD and CV (between brackets) for ETP and TP are 633 ± 146 (23%) and 128 ± 23 (18%), respectively. The median (range) of the thrombogram parameters ETP and TP are 606 nmol.min/L (405 - 1237) and 126 nmol/L (91 - 220), respectively.

Thrombin generation in whole blood of hemophiliac patients

As a proof of concept, whole blood of twenty-seven patients with hemophilia A was activated with 0.5 pmol/L TF and TG was measured. Each blood sample was run in triplicate. The relationship of the thrombogram parameters ETP and thrombin peak height with FVIII levels as measured with the classical aPTT-based one-stage clotting assay are shown in Figure 4. We found statistically significant correlation between plasma FVIII:C activity and the TP height (r = 0.62 95%; CI = 0.3 – 0.81, P = 0.0006) and

between FVIII levels and ETP (r = 0.58 95%; CI = 0.25 – 0.79, P = 0.0014). No correlation was observed between circulating plasma FVIII levels and lag time of WB-CAT (P = 0.17).

To establish that the precision of the assay also holds for whole blood with low FVIII concentrations (3 IU/dL), we performed replicate (N=15) measurements and found a CV value of 10.8% for ETP and 8.0% for TP. To confirm a correlation between FVIII level and ETP and TP, we have measured TF-driven TG in blood from a severe hemophilia A patient (FVIII <1 IU/dL) that was spiked with increasing amounts of recombinant FVIII (Hexilate, CLS Behring). In accordance with an earlier study,[22] using the plasma CAT method, we found a linear relationship between TP and FVIII levels up to 5 IU/dL. Saturation was seen when FVIII levels reached 30 IU/dL. For ETP, the saturation level was reached at FVIII levels higher than 5 IU/dL.

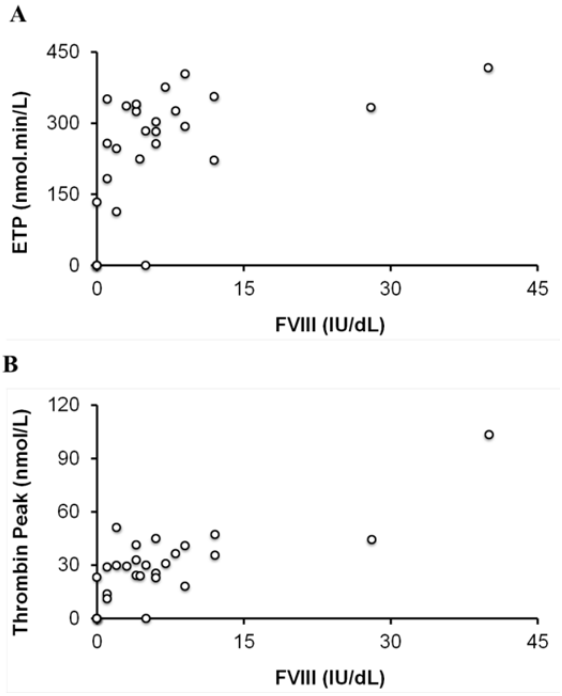

Figure 4. **Whole blood thrombogram parameters and plasma FVIII levels of hemophilia A patients**. Blood from hemophilia patients was activated with 0.5 pmol/L TF and TG was measured as described. Plasma FVIII levels were determined with a one-stage assay. Plots show the FVIII levels and ETP (A) and TP value (B). Thrombogram parameters are the mean of three replicates.

Discussion

Recently, Hemker and co-workers[7] developed a method for continuous monitoring of TG in PPP and PRP. This calibrated, automated and fluorogenic TG assay has multiple potential clinical uses, e.g. estimation of hemorrhagic and thrombotic risk, monitoring of therapy, and detection of circulating procoagulants and anticoagulants.[4, 23] However, of major concern is the need for a centrifugation step to separate plasma from blood cells, which may result in uncontrolled variations in cell counts, activation of blood cells, production of platelet- and monocyte-derived microparticles, and removal of heterologous blood cell aggregates. These variables are known to differentially modulate thrombin generation.[24] Therefore, measuring TG in whole blood is likely approaching (patho)physiological conditions more closely compared to using PPP or PRP and opens the opportunity to measure TG in a point-of-care setting, either at home or in the hospital. Despite reports to the contrary,[10] we consistently found erratic fluorescence signals with this method applied in whole blood because of variable quenching by red blood cells that sediment in the sample holder.

We here report a method that allows the real-time fluorescence monitoring of TG in whole blood. This novel method relates to the use of a thin matrix that contains blood in the interstices of its structure and the use of a rhodamine-based fluorogenic substrate with high quantum yield and which is not consumed significantly during the time of the assay. The excitation and emission wavelengths of the rhodamine substrate have less overlap with the absorption spectrum of hemoglobin compared to the Z-Gly-Gly-Arg-7-amino-4-methylcoumarin substrate used in regular CAT experiments. As a proof of this concept, we demonstrated that the fluorescence data obtained with the WB-CAT method deviated less than 1% from the expected data points. The presence of red blood cells, however, significantly reduces the fluorescence signal but up to a level that is still acceptable. To convert a fluorescence signal into a time course of thrombin concentration three steps are required. First, there should be correction for inner filter effect and substrate exhaustion. It appears however that this is not necessary in the present assay in which a rhodamine 110-based thrombin substrate is used. Second, the contribution of in situ generated α2M-T activity should be subtracted from the raw data. This can be done by a mathematical procedure as described.[6] Finally, the fluorescence change (dFI/dt) should be converted to thrombin concentration. This was done by measuring the linear time dependency of fluorescence intensity of a reaction with a known amount of calibrator

and the thrombin substrate in a whole blood milieu. In addition, calibration is essential because of the varying quenching of fluorescence signal with red blood cell count.

Thrombin generation assays in PPP are often conducted with a rather high amount of TF (5 pmol/L). With this TF concentration less variability in ETP and peak height were reported in comparison with low TF (0.5 pmol/L). However, an important disadvantage of using a high TF concentration is the loss in sensitivity for the intrinsic pathway. That is, high TF concentrations overrate the coagulation pathway that is dependent on factors VIII, IX and XI.[22] Although, the optimal TF concentration for clinical studies has still to be evaluated, we used a low (0.5 pmol/L) TF concentration in the whole blood thrombin generation assay. To avoid interference caused by contact activation in whole blood, we recommend for clinical studies that blood be collected in a tube containing citrate and a FXIIa inhibitor as earlier suggested for measuring TG in plasma.[25, 26]

It is of interest to note that red blood cells contribute directly to TG in whole blood, because these cells are a bigger source of procoagulant cell membranes than those generated by (thrombin) activated blood platelets. Obviously, in view of the antithrombotic action of anti-platelet drugs, it might be concluded that the effects of these drugs probably are produced by inhibiting receptor activation rather inhibiting the formation of procoagulant cell membranes or cell fragments.

The WB-CAT assay showed acceptable intra-assay imprecision of thrombin parameters when blood was triggered with 0.5 pmol/L TF. The inter-individual variation of ETP and TP height were 23% and 18%, respectively. Interestingly, an earlier report showed a similar inter-individual variation in plasma ETP (17.5%).[7] It is apparent that, at least in a group of healthy volunteers, the presence of blood cells does not add up to a larger individual variation of the whole blood TG parameters. We also demonstrated that the ETP and TP height do not vary with hematocrit values higher than 14%.

Our results showed a significant correlation between plasma FVIII levels of patients with hemophilia A and the main whole blood-thrombogram parameters (i.e. ETP and TP values). Despite an overall correlation between WB-CAT and factor concentrations, a certain variation in ETP was observed for FVIII concentrations < 2 IU/dL, suggesting that the FVIII concentration is not the only determinant of TG capacity in hemophiliac patients. We have previously reported that the clinical heterogeneity of bleeding phenotype in patients with FVIII < 1 IU/dL is not associated with their FVIII level, but with the plasma ETP, suggesting the influence of reduced levels of physiological anticoagulants that regulate the coagulation system.[22] The large variations in blood

ETP reported here support our previous findings. These preliminary results suggest the assay is of potential interest for use in patients with hereditary bleeding disorders such as hemophilia. In addition, the whole blood TG assay can be readily developed for application at the point of care to monitor the hemostatic balance in patients with bleeding or thrombotic tendency.

In conclusion, we developed a method that accurately measures whole blood TG using a thin-layer of blood contained in wells of a multi-well microtiter plate. In research settings, this method mimics certain aspects of the *in vivo* situation even better than the existing methods do. Its potential utility in clinical settings needs to be further investigated.

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Chapter 3

Whole Blood Thrombin Generation in Bmal1-Deficient Mice

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Abstract

***Background**:* The Calibrated Automated Thrombogram (CAT) assay that measures thrombin generation (TG) in platelet poor and -rich plasma, is increasingly being recognized as a more sensitive tool to determine the overall function of the hemostatic system. We developed a method enabling the measurement of TG in a small aliquot of blood. The objective was to validate this assay in mouse blood and to examine the rate and extent of TG in a mouse model of premature aging.

Design and Methods: TG was assayed in blood from 20- to 28-week-old *brain and muscle ARNT-like protein-1* (Bmal1)-deficient (knockout, KO) mice and wild-type (WT) littermates. Bmal1-KO mice are known to display symptoms of premature aging. TG was initiated by adding calcium, tissue factor and a thrombin-specific substrate. After TG, the samples were prepared for scanning electron microscopy (SEM).

Results: The intra-assay variations (%) in mouse blood of the endogenous thrombin potential (ETP), peak height, lagtime, time-to-peak and velocity index were 10% or less (n=24). We found that Bmal1-KO mice have a significantly ($p<0.001$) higher ETP (437±7 nM.min; mean±SD, n=7) when compared with WT mice (ETP=220±45 nM.min; mean±SD, n=5). The peak heights also differed significantly ($p=0.027$). By applying SEM we found that Bmal1-KO mice display a denser fibrin network with smaller pores compared to WT mice.

Conclusions: The whole blood TG assay in mice revealed to be reproducible. As a proof-of-principle we have shown that the whole blood TG assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

Introduction

In the research field of thrombosis and hemostasis, the use of mouse models to investigate human coagulation disorders is getting increasingly popular.[1-5] Plasma coagulation tests like the activated partial thromboplastin time (aPTT) and the prothrombin time (PT) are performed on regular basis. However, it has been demonstrated that these tests lack the sensitivity to detect conditions of hypo- and especially hypercoagulability.[6-9] The Calibrated Automated Thrombogram (CAT) assay that measures thrombin generation (TG) in platelet-poor and -rich plasma, is increasingly being recognized as a more sensitive tool to determine the overall function of the hemostatic system.[10-14] Tchaikovski et al.[4] adapted this assay to measure TG in mouse plasma enabling the demonstration of factor (F)V Leiden-, oral contraception- and pregnancy-induced hypercoagulability. In addition, the plasma tests require relatively large amounts of mouse blood and do not account for the presence of blood cells.

Until recently, it was not possible to measure TG in whole blood due to technical problems that lead to highly erratic signals related to quenching of the fluorescent signal and red blood cell sedimentation and retraction.[15, 16] We recently developed a method in which these problems were overcome enabling the measurement of TG in a small aliquot of blood. The advantage of the use of this whole blood CAT (WB-CAT) assay is that it enables us to include the effect of blood cells on TG thereby getting one step closer to physiological conditions compared to plasma. A more practical advantage is that it omits centrifugation steps, thereby also reducing the time needed to perform the assay and the experimental errors that can occur. We recently published the technical validation of this WB-CAT assay demonstrating its utility.[17, 18] An additional advantage of the WB-CAT is the small sample volume necessary to perform the assay (less than 100 µl), rendering it needless to sacrifice the mice.

In this paper we have studied the prothrombotic phenotype in *Brain and muscle ARNT-like protein-1* (Bmal1) clock gene knockout (KO) compared to wild-type (WT) mice using our WB-CAT assay. The Bmal1-KO mice have an impaired circadian behavior and demonstrate loss of rhythmicity in the expression of their clock genes. They are known to have a reduced lifespan and display symptoms of premature aging.[19-21] Plasma clotting assays revealed a discrepancy in test results. Fibrinogen levels and FVII activity were increased and PT was decreased in Bmal1-KO mice, while the aPTT was prolonged.[19, 22, 23]

The goal of this study was to demonstrate that the WB-CAT assay can also be applied on mouse blood. As a proof-of-principle we have shown that the WB-CAT assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

Materials and Methods

Mice

For the first methodical part of the study, whole blood was taken from C57Bl/6 mice (Charles River, Sulzfeld, Germany) to validate the WB-CAT assay (n=6). For the second part of our study, male Bmal1-KO mice and WT littermates were generated in the animal facility of the Centre for Molecular and Vascular Biology (KU Leuven, Leuven, Belgium) as previously described.[23] Their age varied between 22 and 28 weeks (n=12). All animals were kept in 12-hour night/day temperature controlled cages and had free access to drinking water and standard chow (KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium; 13% kcal as fat, caloric value 10.9 kJ/g) *ad libitum*. All animal procedures were approved by the Ethical Committees of the KU Leuven or Maastricht University and performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996).[23]

Blood sampling

Mice were anaesthetized with subcutaneous injection of ketamine and xylazine (0.1 mg/g and 0.02 mg/g body weight). Subsequently, blood was taken via retro-orbital puncture using a glass capillary with a length of 2 cm that was filled with sodium citrate (3.8%). The first three drops of citrate/blood were discarded. The following drops were collected on 3.8% sodium citrate (1/10 volume) in a 0.5 ml eppendorf tube and mixed immediately to prevent clotting. The red blood cell and platelet count were measured using a Cell-Dyn 3200R counter (Abbott Diagnostics, Louvain-La-Neuve, Belgium).

Whole blood thrombin generation

Whole blood TG was performed in a 96-well plate as previously described.[17] Briefly, the total volume of blood needed for TG and calibration measurement in triplicate is 60 µl. For TG measurement, 30 µl of citrated whole blood was mixed with 10 µl thrombin specific substrate (P_2Rho;1.8 mM) and activated with 20 µl tissue factor (TF)

and $CaCl_2$ (1.5 pM and 50 mM, respectively). For the calibration measurement we mixed 30 µl of citrated whole blood with 10 µl thrombin specific substrate (P_2Rho; 1.8 mM) and 20 µl α2M-thrombin (300 nM thrombin activity). Hirudine (50 nM) was also added to the calibrator wells in order to prevent thrombin formation that could affect the linearity of the calibration curve. Immediately after activation, 5 µl of the mixture was added to the wells containing the porous filter paper discs and the wells were covered with 40 µl of mineral oil (USB Corporation) to prevent evaporation. Samples were run for 50 minutes and fluorescence was recorded every 5 seconds with a Fluoroskan Ascent microplate fluorometer with λ_{ex}=485 nm and λ_{em}=538 nm (Thermolabsystems, Helsinki, Finland). All procedures were performed at 37°C.

Scanning electron microscopy

Following TG, the filter paper disks were fixated with gluteraldehyde, dehydrated with ethanol/HMDS and coated with gold using a Rotary-Pumped Sputter Carbon Coater (Quorum Technologies, East Grinstead, West Sussex, England). Samples were analysed using a Phenom G2 pro desktop scanning electron microscopy (SEM, Phenom World, Eindhoven, The Netherlands).

Statistical analysis

The Mann-Whitney U test was used for statistical analysis of the data using SPSS 19.0 for windows (SPSS, Chicago, IL, USA). A p-value below 0.05 was considered to be significant**.**

Results

TF dependency

TG was measured in mouse blood that was activated with various TF concentrations in the presence of P_2Rho (300 µM) and $CaCl_2$ (16.7 mM). The values of the thrombogram parameters are depicted in Table 1. Substantial thrombin generation was already seen in the condition without TF, possibly resulting from the presence of endogenous TF or contact activation. However, addition of 50 µg/ml corn trypsin inhibitor (CTI, Kordia Life Sciences, Leiden, the Netherlands) to the blood collection tube partly reduced contact activation as indicated by a minor prolongation of the lagtime (5.7 vs 7.8 min) and time-to-peak (10.6 vs 13.3 min) without affecting the other parameters. When the

same experiment was performed with mouse plasma, CTI (50 µg/ml) completely diminished thrombin generation. Moreover, in the absence of exogenous TF, thrombin generation was neither observed. These findings indicate that the presence of blood cells diminishes the inhibitory effect of CTI and that the resulting TG is likely not due to the presence of endogenous TF. With increasing TF concentrations, a dose-dependent increase in peak height and decrease in lagtime and time-to-peak was observed. The ETP was not dependent on the TF concentration in this assay.

Table 1: Tissue factor dependency of thrombin generation parameters in C57Bl/6 mice.

	ETP (nM*min)	Peak (nM)	Lagtime (min)	TTP (min)	VI (nM/min)
0 pM TF	310 ± 26	24 ± 0.4	5.7 ± 0.1	10.6 ± 0.1	4.9 ± 0.2
0 pM TF + CTI	339 ± 14	22 ± 0.7	7.8 ± 1.6	13.3 ± 2.0	40 ± 0.4
0.25 pM TF	284 ± 26	30 ± 2.5	4.3 ± 0.3	7.4 ± 0.3	9.8 ± 0.9
0.5 pM TF	285 ± 12	44 ± 4.3	3.0 ± 0.2	5.3 ± 0.3	19 ± 2.5
1 pM TF	320 ± 11	55 ± 3.1	2.2 ± 0.1	4.2 ± 0.1	27 ± 2.0
2 pM TF	345 ± 14	68 ± 7.4	1.5 ± 0.1	3.2 ± 0.1	42 ± 8.5
2.5 pM TF	332 ± 16	80 ± 2.1	1.1 ± 0.1	2.7 ± 0.1	52 ± 2.6

Data represent the mean ± SD (N=3); TF, tissue factor; CTI, corn trypsin inhibitor; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index.

Precision of the WB-CAT assay in mouse blood

The intra-assay variation of the WB-CAT assay was tested for 24 replicates. Blood was taken from three C57Bl/6 mice and activated with 0.5 pM TF, 300 µM P_2Rho and 16.7 mM $CaCl_2$. The mean intra-assay variation of the ETP, peak height, lagtime, time-to-peak and velocity index from one representative mouse are shown in Table 2. Except for the ETP, all the parameters have an acceptable coefficient of variation (CV ≤10%).

Table 2: Intra-assay variation of whole blood thrombin generation in a C57Bl/6 mouse.

	Mean	SD	CV
ETP (nM*min)	309.7	37.1	12.0
Peak (nM)	40.3	3.1	7.6
Lagtime (min)	2.6	0.2	7.4
TTP (min)	5.0	0.3	6.2
VI (nM/min)	16.8	1.7	10.1

Blood was activated with 0.5 pM TF and 16.7 mM $CaCl_2$. SD, standard deviation; CV, coefficient of variation; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index; The sample was tested 24 times in one single run.

Comparing TG in Bmal1-KO and WT mice

TG was measured in blood of WT and KO mice in the presence of 300 µM P_2Rho, 0.5 pM TF and 16.7 mM $CaCl_2$. The mean age of the WT and KO mice was 24.2 and 25.2 weeks, respectively (p=0.48). Figure 1 shows representative TG curves of the WT group (black line) and the KO group (interrupted line). The mean values of the TG parameters for each group are depicted in Table 3. The lagtime, time-to-peak and velocity index did not differ significantly between the two groups (p-values were 0.45, 0.145 and 0.742 respectively). However, significant higher TG peak height (30.1 nM vs 45.8 nM; p=0.027) and ETP (220 nM.min vs 437.6 nM.min; p<0.001) values were found in the KO mice. The higher TG in the KO mice correlates with the prothrombotic phenotype seen in these mice. Red blood cell count was found to be significantly (p<0.001) higher in the KO mice ($9914x10^3$/µl ± $724x10^3$/µl) compared to WT mice ($7900x10^3$/µl ± $334x10^3$/µl). Also the platelet count of KO mice was significantly (p<0.005) higher ($1281x10^3$/µl ± $157x10^3$/µl) compared to that of WT mice $942x10^3$/µl ± $152x10^3$/µl).

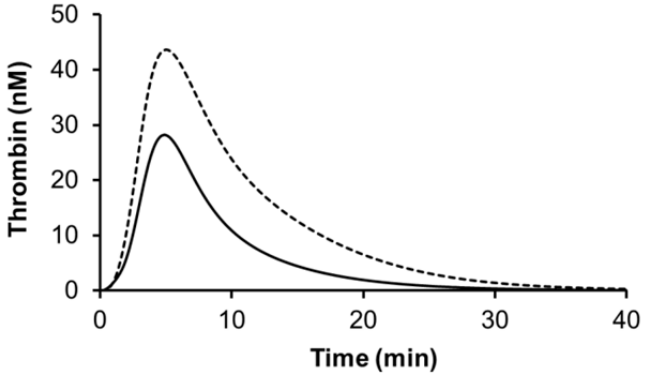

Figure 1: Representative TG curves of Bmal1-KO and WT mice. Samples were activated with 0.5 pM TF and 16.7 mM calcium. The full and interrupted lines represent the WT and KO mice, respectively. Both curves are the mean of 3 measurements.

Table 3: Whole blood thrombin generation parameters in Bmal1-KO and WT mice.

	WT		KO		
	Mean	**SD**	**Mean**	**SD**	**P-value**
ETP (nM*min)	220.3	45.1	437.6	78.3	**<0.001**
Peak (nM)	30.1	3.7	45.8	14.3	**0.027**
Lagtime (min)	2.5	0.8	2.2	0.5	0.45
TTP (min)	4.8	0.9	5.9	1.6	0.145
VI (nM/min)	13.7	3.3	15.0	9.6	0.742

Blood samples of Bmal1-KO and WT mice were activated with 0.5 pM TF and 16.7 mM CaCl2. SD, standard deviation; ETP, endogenous thrombin potential; TTP, time-to-peak; VI, velocity index; KO, n=7; WT, n=5.

Visualization of fibrin network

At the end of the TG assay, the samples were fixated and analysed by scanning electron microscopy. Representative images are shown in Figure 2 (5000x magnification). The images in the upper part are clots from WT mice, while images of the lower part represent those from the KO mice. While the fibrin structure in WT clots is more localized, the fibrin formation is seen throughout the whole paper matrix in KO clots. In addition, much more fibrin is present in clots of KO versus WT mice.

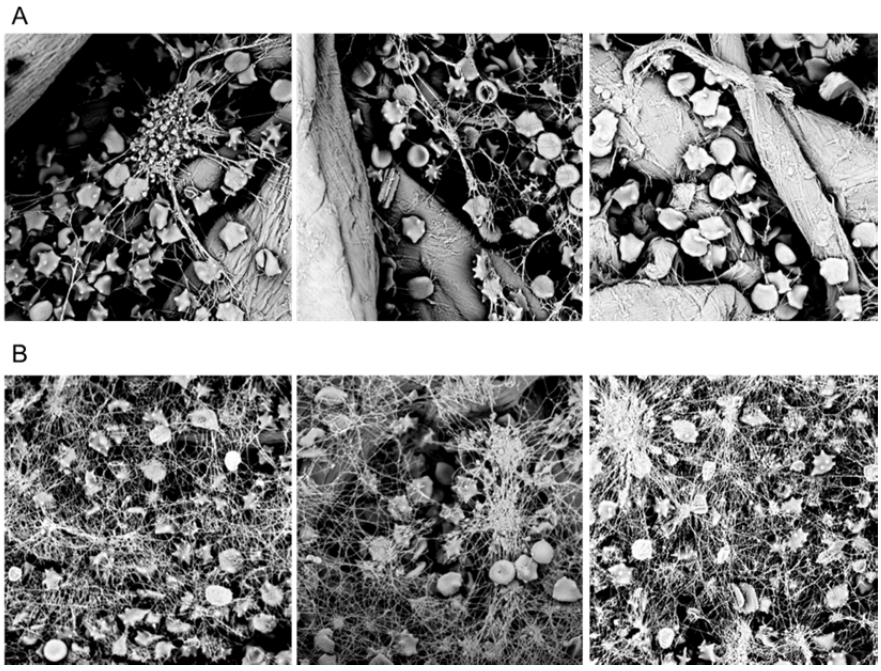

Figure 2: Representative SEM pictures of the fibrin network of Bmal1-KO and WT mice. TG was activated as described in Figure 1. After fixation SEM pictures were taken of clots of Bmal1-WT (A) and KO-mice (B). Magnification was 5000x.

Discussion

It is becoming increasingly apparent that global blood clotting tests like thrombin generation assays are more sensitive to detect conditions of hypo- and especially hypercoagulability when compared to conventional assays only measuring clotting times. Since transgenic mouse models have been very helpful in increasing our insights in the process of blood coagulation, it is appropriate to develop a method by which TG can be measured in mouse blood.

TG tests have been reported for platelet-poor and -rich murine plasma. However, a major drawback of these assays is the requirement of relatively large amounts of plasma implying that the animal has to be sacrificed. In addition, under the conventional CAT conditions, mouse plasma appears to be highly reactive hampering reliable TG measurements.[1, 4] These problems were solved by diluting the plasma samples. However, dilution of plasma dramatically distorts the balance between pro-

and anticoagulant activity of the sample.[11] Our assay proved to be reproducible and requires only a small amount of blood sample (60 µl per assay) and enables us to perform TG in blood collected at different time-points from the same mouse. Moreover, the usage of whole blood eliminates the need to separate the analyte with a centrifugation step thereby reducing the number of pre-analytical variables and the experimental errors. Finally, measuring TG in the presence of blood cells might bring the assay closer to physiology. It is advised to use a low TF concentration (0.5 pM) to keep the assay sensitive for the coagulations factors of the intrinsic coagulation pathway and to prevent clotting before the measurement is actually started.

As proof-of-principle we measured TG in the Bmal1 clock gene KO mice, which display symptoms of premature aging.[21-23] These KO mice exhibit imbalances in the haemostatic mechanism, i.e. higher plasma levels of procoagulant factors VII, VIII, fibrinogen and raised platelet count on the one hand and decreased endothelial anticoagulants (thrombomodulin and protein C receptor) on the other hand. It was concluded that, with aging, progressive endothelial dysfunction, rising platelet counts, and high factor VII further enhance thrombogenicity and provoke priapism.[23] In this paper we showed that Bmal1-KO mice have a higher TG and fibrin formation compared to their Bmal1-WT littermates. This is confirming the results of Wolberg et al., who established that the fibrin formation is dependent on the thrombin concentration. A low thrombin concentration leads to a loosely woven fiber network with thick fibrin fibers, whereas high thrombin concentrations lead to a compact, dense network of thin fibrin fibers.[24-26] We also confirmed a higher platelet count in Bmal1-KO mice compared to WT mice of the same age. However, earlier studies from our group demonstrated that the whole blood platelet count does not contribute to the extent of thrombin generation in whole blood.[17] Additionally, we established a higher red blood cell count in KO compared to WT mice. Based on our previous study it is not likely that an increased hematocrit affects TG in our assay.[17] This is in contrast to the findings of Whelihan et al., who did find a hematocrit-dependent TG in mixtures containing only plasma and red blood cells.[27-29] However, our experimental conditions were completely different (lower TF concentration; creation of a thin layer of blood by means of a porous matrix) which may provide an explanation for the observed differences.

The present findings of our study showed that our whole blood thrombin generation assay is also applicable on mouse blood and has a good reproducibility. As a proof-of-

principle we have shown that the whole blood TG assay is capable of detecting a prothrombotic phenotype in Bmal1-KO mice.

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Chapter 4

Hypoxia induces a prothrombotic state: results from the “redmeetswhite” study

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Submitted.

Abstract

Introduction: Hypoxia, a pathological condition in which (a part) of the body is deprived of an adequate oxygen supply, is directly associated with deep vein thrombosis and venous thromboembolism. The circumstance of this hypoxia-induced prothrombotic phenotype likely lies in a derailed hemostatic process of which the precise defect remains to be clarified. We performed a high-altitude study in the Mont Blanc massive in an attempt to get a better insight in how the process of blood coagulation is affected when suffering from hypoxia.

Methods: Two groups of 15 healthy individuals each gradually went up to an altitude of 3900 meters either by climbing (active group) or by cable car (passive group). Both groups were assayed for plasma Von Willebrand Factor (VWF) and factor VIII (FVIII) levels, thrombin generating capacity in plasma and whole blood, heart rate, oxygen saturation levels and blood pressure.

Results: Blood pressure was not affected by changes in altitude. Oxygen saturation levels decreased with increasing altitudes for both groups and VWF and FVIII levels increased significantly only in the active group. Thrombin generation assays in plasma revealed contrasting results between the two groups. At 2045 meters, thrombin generation increased in the active group, while it decreased in the passive group. Thrombin generation in whole blood revealed that in both the active and the passive group, hypoxia was associated with an increase in peak height, endogenous thrombin potential (ETP) and velocity index and a decrease in lagtime and time-to-peak. Interestingly, these opposing results between whole blood and plasma indicate that the cellular portion of the blood is likely responsible for the prothrombotic state. The increase seen in plasma thrombin generation of the active group could be explained by the significant increase in FVIII and VWF levels.

Conclusion: We found by applying thrombin generation in whole blood, strong indications that hypoxia induces a prothrombotic state. Together with an increase in FVIII and VWF, we have strong indications that cellular driven thrombin generation is at least partially responsible for the association between hypoxia and venous thrombosis.

Introduction

Hypoxia is an imbalance between oxygen supply and consumption. Strenuous physical exercise and/or reduction of the normal oxygen supply (hypoxia) will affect cellular viability and can lead to cellular dysfunction, cell death and (multiple) organ failure.[1-3] The most hypoxia-sensitive organ is the brain, which has a high need for oxygen.[2, 4] Hypoxia is known to induce an increase in platelet aggregation and activation of blood coagulation, resulting in thrombus formation.[5, 6] Fragmentation of a venous thrombus might cause pulmonary embolism or stroke.[2]

In order to study the influence of hypoxia on hemostasis, several studies have been done in healthy individuals being exposed to lower oxygen pressure by either going to high altitude or by inducing hypobaric hypoxia. It is well known that hypobaric hypoxia due to air travel leads to the development of venous thrombosis.[7] The odd's ratio varies between 2- and 6-fold depending of the positions of the seat in the plane, obesity, duration and number of flights, gender, age, length, usage of oral contraceptives, sleeping and coagulation defects (e.g. Factor V Leiden, prothrombin mutation, high factor VIII and IX levels, …).[6, 8] However, the most relevant element that gives the absolute risk for the development of symptomatic venous thrombosis after long-hour travel is low (1/5000) and still unknown.[6] In contrast to these results, there are also studies that showed an association between hypoxia and a reduced coagulation or even that hypoxia does not have any effect at all.[9-12]

In previous studies two conceptual errors were made that might explain the conflicting data. First, on high altitude the change in barometric pressure has an effect on blood drawing when using the vacutainer system, as less blood will enter the tube, while the amount of anticoagulant remains constant.[13] In other words, the ratio blood:anticoagulant is no longer correct and thereby induces an artificial anticoagulant effect. Another conceptual error often made is that exercise, e.g. due to climbing or walking to a higher altitude induces an increase in Von Willebrand Factor and factor VIII (FVIII), causing an exercise-increased hemostatic response leading to a prothrombotic phenotype.[14-17]

Our aim was to investigate the effect of hypoxia on hemostasis avoiding these two conceptual errors. We formed two groups: one that climbed to high altitude (3900 meters) and another that was transported to high altitude. In addition, blood coagulation was studied by assaying the thrombin generating capacity in plasma and

whole blood, rather than performing blood clotting test that are less sensitive to changes in the blood clotting status of a subject.[18]

Materials and Methods

Study protocol

We performed a randomized controlled study to investigate the effect of oxygen deprivation on coagulation. Healthy volunteers that wanted to participate in our study had to pass a physical checkup including medical history, complete physical examination, an electrocardiogram and exercise test. Exclusion criteria were: medication interfering with coagulation, a history of cardiovascular disease, having a mobility impairment (not being able of walking by themselves or on a normal manner without discomfort for the subject), being younger than 18 years or older than 50 years and not meeting demands of physical checkup. Healthy volunteers with climbing experience (N = 30) were included and randomized into either of two groups after age and gender stratification, respectively. Volunteers were separated in two groups: one of 18-35 years old subjects and one of 36-50 years old subjects. These two groups were again divided into two separate groups according to gender (men and women). For each of the 4 groups a random sequence of two letters “A” and “B” (e.g. ABBAAABBAB) was written by an independent person. The first volunteer of the group got the first letter of the sequence, the next volunteer of the same group got the following letter of that sequence. Consequently, two groups were formed that were stratified on the basis of their age and gender. The first group was the “active” climbing group that increased in altitude by actively climbing (group A). The second group was the “passive” group that increased in altitude by cable car (group B). The formation of these two groups was done in order to investigate the role of exercise-induced increase in Von Willebrand Factor and FVIII, rather than altitude-induced hypoxia, as a cause for a hypercoagulable state. Blood samples were taken at different heights: at 50 meters, 1100 meters, 2045 meters, 3100 meters and 3900 meters. Blood drawing and testing was done on the same day for both groups to exclude day to day variability. Blood pressure, heart rate and blood oxygen saturation levels were recorded by trained medical personnel at each testing day. The volunteers had to complete a questionnaire every testing day about their physical and mental condition (according to the Lake Louise Consensus on the Definition of Altitude Illness). In total,

the study lasted 8 days. Two participants (one of each group) had to leave the study at 3100 meters of altitude as they suffered from acute mountain sickness. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre and by the local authorities. The study met all institutional ethics requirements according to the Helsinki declaration (2008).

Blood collection

Blood was taken from 30 healthy adult volunteers after obtaining their written informed consent. Blood (9 volumes) was aseptically drawn in vacutainer tubes (Greiner Bio-One) containing 3.2% sodium citrate (1 volume), from the antecubital vein of healthy subjects. The blood was kept at room temperature (± 22°C) and used within 2 hours. Platelet poor plasma was obtained by centrifugation twice at 2630 × g for 10 min. Plasma samples were snap-frozen on dry ice and put into the -80°C freezer. Von Willebrand Factor and FVIII concentrations in plasma were measured with the STA-R evolution (Diagnostica Stago). At higher altitudes blood collection tubes were not completely filled with blood due to the lower barometrical pressure. The mean (±SD) content of the tubes were as follows: 0.9 ml of citrate plus 8.1±0.05 ml of blood at 50 meters, 8±0.06 ml of blood at 1100 meters, 7.7±0.07 ml of blood at 2045 meters, 7.2±0.08 ml of blood at 3100 meters and 7±0.09 ml of blood at 3900 meters (Figure 1). The corresponding blood percentages in the tubes were respectively 90%, 89.9%, 89.5%, 88.9% and 88.6%. At the highest altitude, the blood content was 1.4% less than compared to sea level, which induces a negligible dilution effect.

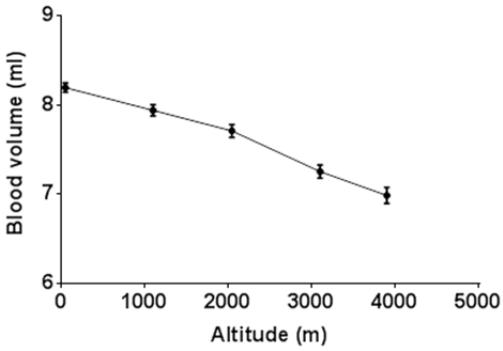

Figure 1: Blood tube volumes at different altitudes. The volume capacity of the blood collection tubes were measured on each altitude 10 times to investigate the effect of lower barometrical pressure on tube filling. Data are mean values with SD.

Thrombin generation in plasma

Thrombin generation in plasma was measured with the Calibrated Automated Thrombogram assay as previously described.[17] Briefly, 80 µl of platelet poor plasma was mixed with 20 µl of a mixture containing 6 pM tissue factor (TF, Dade-Behring) and 24 µM phospholipid vesicles (DOPS/DOPC/DOPE; 20/60/20 mol/mol/mol; Avanti). After 5 minutes of incubation at 37°C, thrombin generation was started with 20 µl of the activator containing 100 mM $CaCl_2$ and 2.5 mM of the thrombin-specific substrate, the Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (Bachem). Fluorescence was measured with a Fluoroscan Ascent reader (Thermo Labsystems). Samples were run in triplicate and calibration of each curve was done to correct for inner-filter effects and substrate consumption. All procedures were performed at 37°C and data were analyzed with dedicated software (Thrombinoscope, Stago).

Whole blood thrombin generation

Whole blood thrombin generation was performed in a 96-well plate as previously described.[19] Briefly, 30 µl of citrated whole blood was mixed with 10 µl thrombin specific substrate (P_2Rho; 1.8 mM), and activated with either 20 µl TF and $CaCl_2$ (1.5 pM and 50 mM, respectively) or 20 µl human thrombin calibrator (α_2M-T, 300 nM thrombin activity). Immediately after mixing, a sample of 5 µL was added to paper disks (Whatman GmbH) and covered with 40 µl of mineral oil (USB Corporation) to prevent evaporation. Samples were run for 50 minutes and fluorescence was recorded every 6 seconds with a Fluoroskan Ascent microplate fluorometer with λ_{ex} = 485 nm and λ_{em} = 538 nm (Thermolabsystems). Samples were run in triplicate and calibrated as previously described.[20] All procedures were performed at 37°C and thrombin generation curves were calculated as previously described.[21]

Statistical analysis

The Wilcoxon matched-pairs signed rank test was used for significance testing. P-values <0.05 were considered to be statistically significant. Data given are medians [interquartile ranges] unless otherwise indicated.

Results

Effect of altitude on the amount of blood entering the vacutainer

The blood tubes were not completely filled with blood at higher altitudes resulting in a slightly higher citrate concentration in these tubes compared to baseline at 50 meters. Therefore, we performed a control experiment in which we investigated whether we had to correct for these higher citrate concentrations in our assays. Blood taken from a healthy donor was supplemented *in vitro* with citrate up to the same concentration as on 50, 3100 and 3900 meters of altitude and we also used two higher concentrations that corresponded to 4400 meters and 4660 meters of altitude. Thrombin generation in plasma and whole blood were not affected by the increase of citrate at the altitudes on which blood samples were taken for this study (Figure 2).

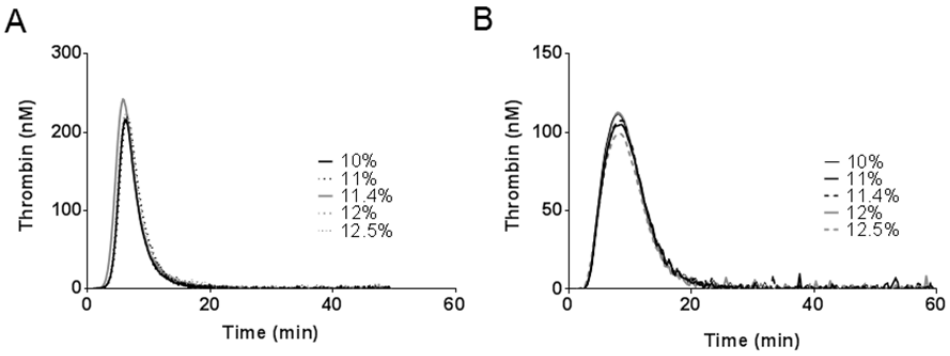

Figure 2: Thrombin generation measured in whole blood containing various amounts of citrate. Thrombin generation was measured in recalcified whole blood (A) and plasma (B) of samples containing varying citrate concentrations. Whole blood thrombin generation was measured with 0.5 pM TF and 16.7 mM $CaCl_2$, while thrombin generation in plasma was activated with 1 pM TF, 4 µM phospholipid vesicles and 16.7 mM $CaCl_2$. Samples were run in triplicate and the mean values of the thrombin generation curves are depicted.

Probably the excessive amount of calcium used in our assay already corrects for small differences in citrate concentration in our samples. A small decrease in plasma thrombin generation was observed in the highest citrate concentration corresponding to approximately 4660 meters of altitude, indicating that from that point onward the plasma-based thrombin generation assay becomes sensitive to the increased citrate concentration. The relation between barometric pressure and altitude or oxygen saturation levels in the active and passive group is depicted in figure 3.

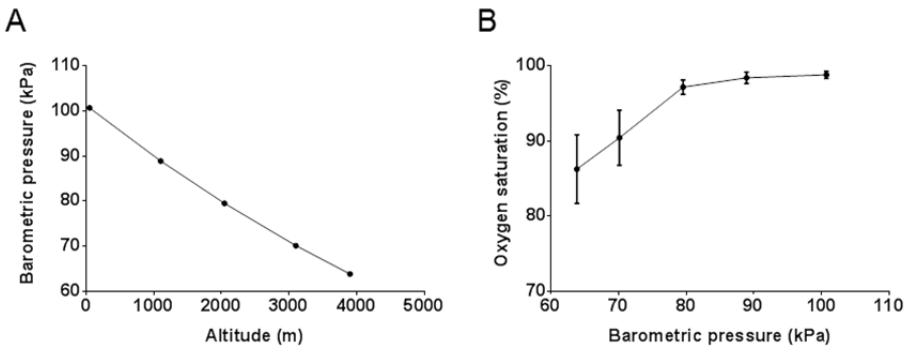

Figure 3: Barometric pressure on different altitudes and oxygen saturation. (A) Relation between barometric pressure and different altitude levels. (B) The oxygen saturation (%) for both groups measured at different barometric pressures.

Table 1: Basic characteristics of both groups at 50 m (day 1). Data are mean values ± SD.

Baseline	Active group	Passive group
Gender (male/female)	7/8	7/8
Age (years)	31.7 ± 5.7	28.0 ± 5.2
Hematocrit (%)	41.3 ± 5.1	41.6 ± 3.3
Hemoglobin (g/L)	8.7 ± 1.1	8.8 ± 0.7
Heart rate (min^{-1})	69 ± 13.6	67 ± 10.9

Effect of altitude-induced hypoxia on oxygen saturation levels, heart rate and blood pressure

There were no differences between the active group A and the passive group B in baseline characteristics (Table 1). Measurement of the oxygen saturation levels in capillary blood revealed that both groups suffered from hypoxia to the same extent starting at 2045 meters of altitude, the median [interquartile ranges] was 97% [97%-98%] for the active group and 97% [97-97] for the passive group (Figure 4A, p-values were <0.0001 for both groups compared to baseline at 50 meters 99% [99-99]). The oxygen saturation levels were lower at 3900 meters: 84% [81-91] for the active group and 87% [84%-90%] for the passive group. Adaptation of the body to hypoxia by increasing heart rate started at 2045 meters for the active group (77/min [70-93], p=0.0129) in contrast to the passive group that showed an increased heart rate from 3100 meters of altitude (85/min [74-90], p=0.0004) compared to baseline values 68/min [59-74] (Figure 4B).

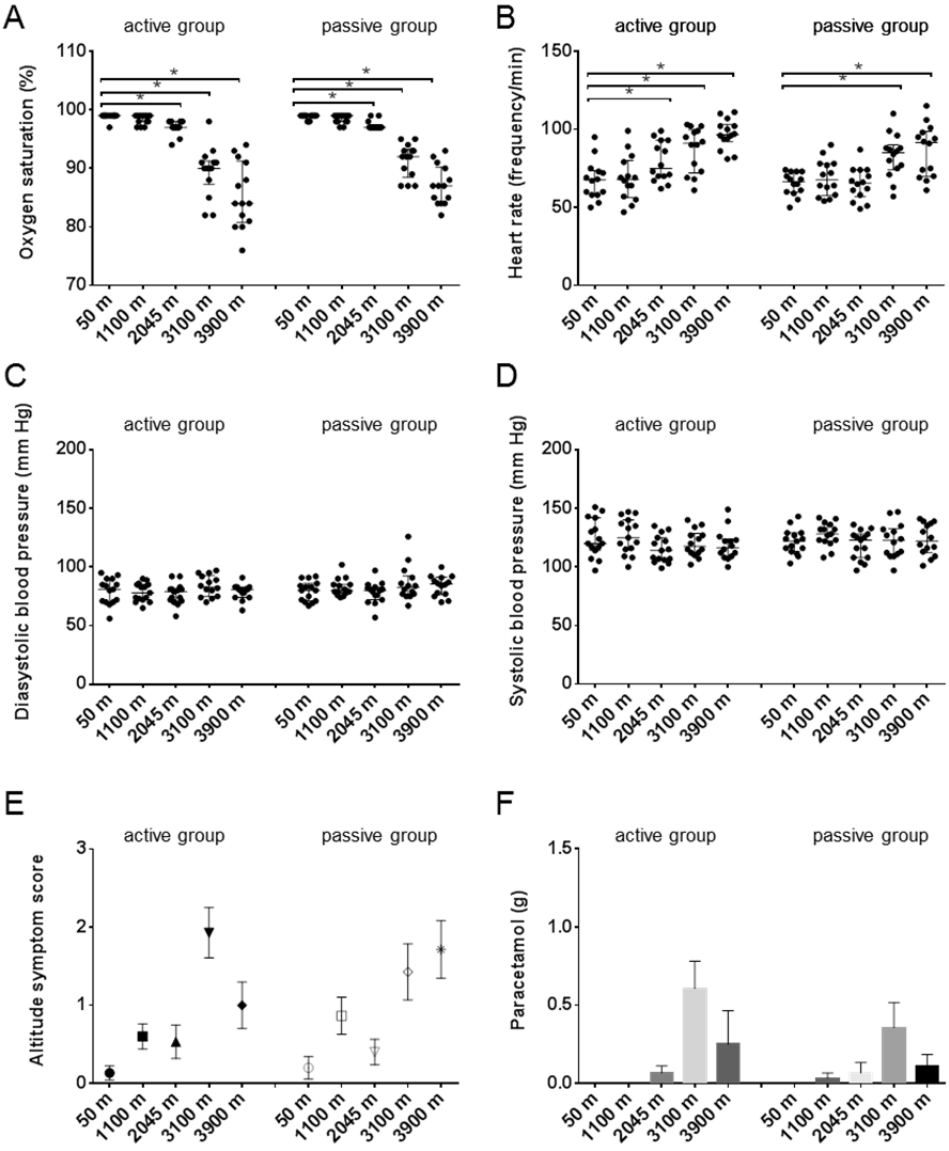

Figure 4: Effect of hypoxia on oxygen saturation, heart rate, blood pressure, altitude symptom score and paracetamol intake. Data represent medians with interquartile ranges (A-D) or mean with SEM (E-F). *p<0.05

Systolic and diastolic blood pressures were not affected by altitude (Figure 4C and D). To detect early signs of altitude sickness, the participants completed a questionnaire regarding altitude sickness on each testing day at every altitude (based on the Lake Louise Consensus on the Definition of Altitude Illness). We found an increase in symptoms related to altitude sickness with increasing altitude up to 3900 meters (Figure 4E). In addition, intake of paracetamol showed the same trend up to 3100 meters (Figure 4F). The lower intake of paracetamol at 3900 meters might be related to the fact that the testing day at 3900 meters was the last day of the study and people knew they were descending.

Von Willebrand Factor and FVIII levels in plasma

Measuring Von Willebrand Factor levels in plasma revealed a gradual increase in the active group starting from 2045 meters (126% [107-149] compared to 104% [92-118] at baseline) (Figure 5A). Similar results were obtained for FVIII levels, as they increased significantly in the active group from 125% [111-152] at baseline up to 156 [129-179] at 2045 meters (Figure 5B). At 3100 and 3900 meters FVIII levels were not significantly different compared to baseline values. In the passive group the Von Willebrand Factor and FVIII level were only increased at the highest altitude (Von Willebrand Factor increased from 98% [78-113] at baseline up to 102% [94-117] and FVIII increased from 108% [102-118] up to 119% [98-144).

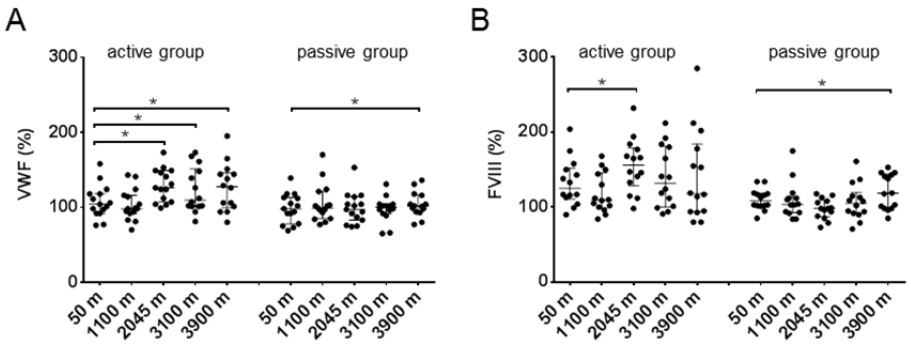

Figure 5: Von Willebrand Factor (A) and FVIII (B) levels in plasma. Data are medians with interquartile ranges. *p<0.05

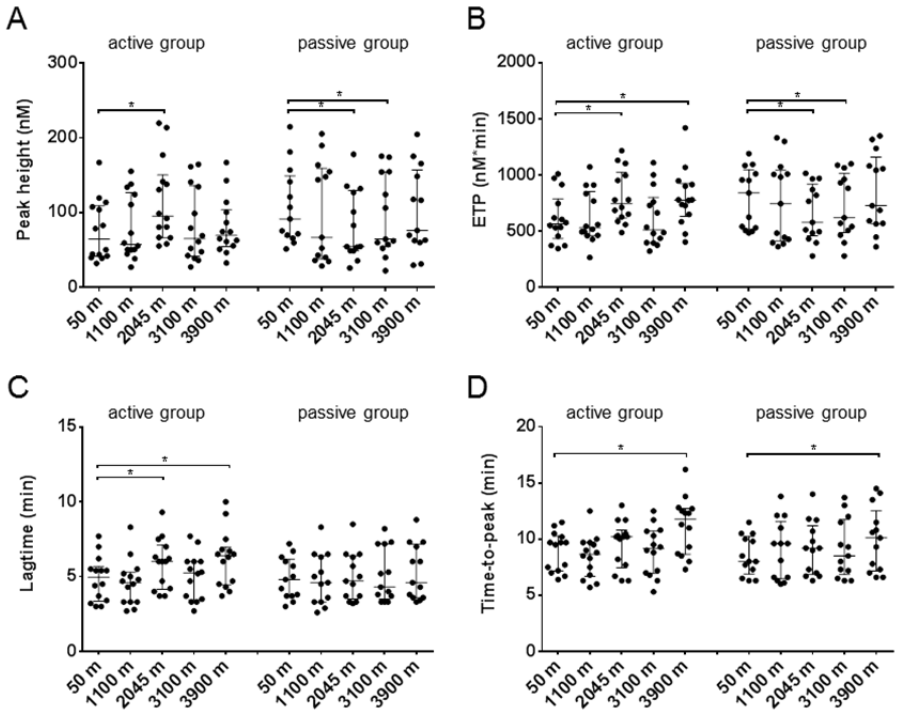

Figure 6: Thrombin generation in plasma. Thrombin generation was started with 1 pM TF, 4 μM phospholipid vesicles and 16.7 mM $CaCl_2$. The parameters depicted are the Peak height (A), ETP (B), Lagtime (C) and Time-to-peak (D). Data are medians with interquartile ranges.

Thrombin generation in plasma

To investigate a possible association between hypoxia and blood coagulation we applied thrombin generation in platelet poor plasma and whole blood. Thrombin generation measured in platelet poor plasma revealed an increase in peak height for the active group from 65 nM [42-109] at baseline up to 96 nM [67-150] at 2045 meters versus a decrease for the passive group from 92 nM [68-149] to 55 nM [49-130] (Figure 6). Similar results were found for the ETP in both groups. In the active group, the ETP increased from 565 nM*min [437-790] at baseline to 746.3 nM*min [619-1024] at 2045 meters, while in the passive group the same route was accompanied with a decrease in ETP from 841 nM*min [519-1049] to 580 nM*min [460-919] ($p<0.02$). Surprisingly, the lagtime and time-to-peak were prolonged for both groups at the highest altitude (e.g. time-to-peak was 11.8 min [8.7-12.7] versus 9.6 min [7.2-10.3] at baseline for the active group and 10.1 [7.2-12.5] versus 8 min [6.9-10.3] at baseline for the passive group; p-values <0.005). These results indicate that the increase seen in

plasma peak height, ETP, FVIII and Von Willebrand Factor in the active group at 2045 meters is predominantly caused by exercise.

Thrombin generation in whole blood

Recently we developed a method to detect thrombin generation in whole blood which has a major advantage as it also takes the cellular portion of the blood into account. In contrast to the results seen with thrombin generation in plasma, thrombin generation in whole blood revealed a significant increase in both groups for peak height, ETP and velocity index, related to the increase in altitude. The peak value of the active group was 158 nM [105-182] at baseline and increased up to 215 nM [188-279] at 3100 meters (p=0.0006; Figure 7A). The peak height of the passive group increased from 143 nM [114-160] at baseline up to 249 nM [196-276] at 2045 meters (p=0.0005). The ETP was increased from 747 nM*min [578-965] at baseline up to 1085 nM*min [723-1308] for the active group (p=0.0166) and from 749 nM*min up to 1098 nM*min for the passive group at 2045 meters (p= 0.0005; Figure 7B). The lagtime and time-to-peak were shortened with increasing altitude. The lagtime of the active group decreased from 4 min [3.95-4.1] at baseline down to 3.45 min [3.3-4.0] at 3900 meters (p=0.0002) and for the passive group from 4 min [3.8-4.0] down to 3.4 min [3.15-3.7] at 3100 meters (p=0.0017; Figure 7C). The time-to-peak of the active group decreased from 9 min [8.1-10.4] at baseline down to 6.2 min [5.8-6.6] at 3900 meters (p=0.0001) and for the passive group from 8.9 min [8.5-10] down to 6.1 min [5.9-6.8] at 3100 meters (p=0.0002; Figure 7D). The velocity index, which is a measure for the velocity of thrombin formation, increased from 31.2 nM/min [17-43] at baseline up to 78 nM/min [67-107] at 3100 meters for the active group and from 25 nM/min [24-31] up to 84 nM/min [66-101] at 2045 meters for the passive group. Since the prothrombotic phenotype was observed in both the active and the passive group, our results indicate that this effect is predominantly caused by hypoxia and not by exercise. Although all parameters were significantly different from baseline at the highest altitude (3900 meters), the influence of hypoxia on these parameters seems to already achieve a maximal effect at 3100 meters.

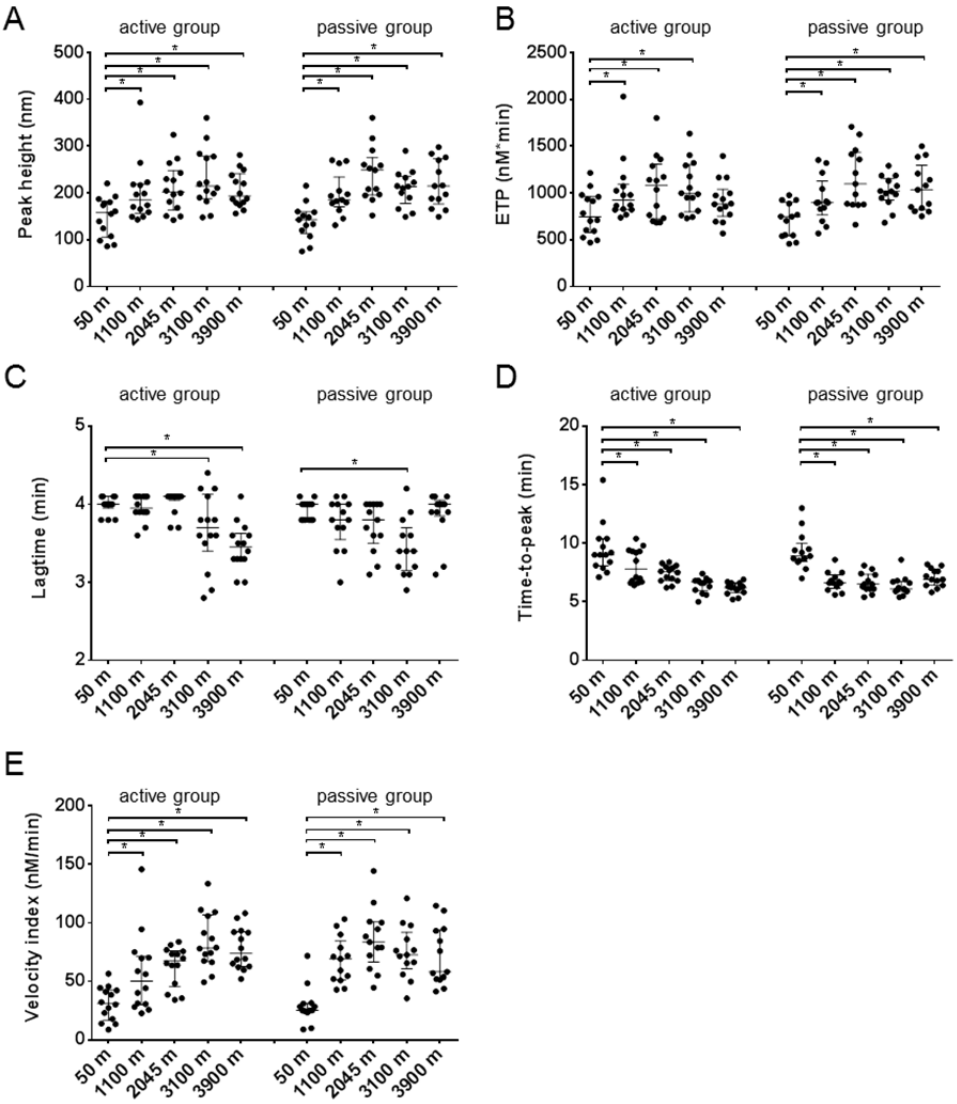

Figure 7: Thrombin generation in whole blood. Thrombin generation was started with 0.5 pM TF and 16.7 mM $CaCl_2$. The parameters depicted are the Peak height (A), ETP (B), Lagtime (C), Time-to-peak (D) and Velocity index (E). Data are medians with interquartile ranges.

Discussion

Hypoxia has been indicated to be associated with venous thromboembolism.[5, 22] Several studies have been done to investigate the adverse effects of hypoxia on the human body. Contrasting results were found in these studies, but mostly a prothrombotic phenotype was found.[9-12] Many studies included persons that reached certain altitudes actively and it is known that physical activity induces a prothrombotic phenotype. We decided to form two groups, an active and a passive group in order to dissect coagulation effects induced by altitude from those induced by exercise and altitude. Our finding that the plasma FVIII and Von Willebrand Factor concentration increased with increasing altitude in the active group but not in the passive group supports our assumption. Using the thrombin generation assay, which is an established sensitive method in detecting hypo- and hypercoagulability states,[17, 18, 23-25] we demonstrated that thrombin generation increased at 2045 meters in the active group, but decreased in the passive groups. The increase seen in the active group is accompanied with an increase in FVIII and Von Willebrand Factor, and is therefore most probably exercise-induced. Thrombin generation measured in whole blood revealed that in both groups peak height, ETP and velocity index increased gradually with increasing altitude, while the lagtime and time-to-peak decreased. Since thrombin generation in whole blood increased and accelerated with increasing altitude similarly in both groups, this prothrombotic effect is induced by hypobaric hypoxia and is not related to exercise. This observation is in agreement with the findings of Schreijer et al.[8] Apparently, not the plasma but the cellular part of the blood is responsible for the prothrombotic phenotype observed in the whole blood thrombin generation.

Rosendaal et al.[26] published that all long-duration travel, either by car, bus or airplane, is associated with an increased risk of venous thrombosis. Nevertheless, traveling by plane was associated with the highest risk (up to 6 times increased risk) indicating a role for hypobaric hypoxia.[6, 8, 26-28] In contrast, Toff et al.[29] performed a study in which healthy volunteers were exposed to hypobaric hypoxia resembling the air pressure in the flight cabin. Interestingly they did not find any relation between hypoxia and plasma markers such as thrombin-antithrombin complexes, Von Willebrand Factor, d-dimer and plasma thrombin generation. This is in line with our study as the participants in our study only had reduced oxygen saturation levels saturation values at 3100 meters, where the barometric pressure is lower than the cabin pressure during flight (which usually resembles to a stay at 1500-2500 meters of

altitude). In our study we induced hypoxia by increasing in altitude up to 3900 meters corresponding to 12.7% of oxygen level compared to 21% at sea level. We observed that from 2045 meters, oxygen saturation levels measured from capillary blood decreased progressively down to 84% [81-91] in the active group and 87% [84-90] in the passive group.

On higher altitudes, blood tubes do not fill completely and it takes a longer time to be filled. In our study, the percentage of blood in the tubes decreased for 1.4% at the highest altitude. This dilution is only minimal and it will not affect coagulation. However, since the volume of the anticoagulant, the sodium citrate, remained the same it could induce an artificial anticoagulant effect. We found that from approximately 4660 meters of altitude this phenomenon is able to influence hemostatic assays such as thrombin generation in plasma. As our study did not exceed 4000 meters of altitude, there was no need to correct for it. Studies that exceed 4660 meters should either correct for the reduced amount of blood in the tubes or draw the blood by a method which is not based on vacuum.

One aspect that we did not investigate is hypoxia/reoxygenation and its effect on hemostatic parameters. Recently Brill et al.[30] performed a study in which mice were kept in a hypoxic environment (6% oxygen) for 24 hours followed by a reoxygenation period. They found that after induction of 1 hour stenosis of the inferior vena cava, thrombus size and prevalence was dramatically increased in these mice compared to control animals that were kept at normoxic conditions.[30] There are also several case reports known of patients that suffered from venous thrombosis when returning from the mountains or after air travel. Whether these thrombi were formed during or after hypobaric pressure (either in plane or mountains) is not known. Interestingly using ultrasound, Rosendaal and coworkers found asymptomatic thrombi in passengers after air travel indicating that these thrombi were formed during hypobaric situations.[26]

In conclusion we have found clear proof that hypoxia is associated with an increased thrombin generation in whole blood, irrespective of the group (active or passive). As we did not find a comparable increase in thrombin generation in the plasma of the passive group, we have strong indications that the cellular part of blood contributes to this prothrombotic phenomenon.

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Chapter 5

Thrombin Generation Assay using Factor IXa as a Trigger to Quantify Accurately Factor VIII Levels in Haemophilia A

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Abstract

Background: The available methods for measuring factor VIII (FVIII) activity suffer reportedly from lack of sensitivity and precision in the <1 IU/dL range. This precludes correlation of clinical phenotype with FVIII levels.

Objectives: To study a possible association between clinical phenotype in patients with FVIII levels <1 IU/dL.

Methods/Results: The FIXa-driven FVIII assay (FVIII-CAT) has a detection limit of 0.05 IU/dL. For the range of 0-2 IU/dL FVIII, the intra-assay coefficient of variation (CV) is around 2% and the inter-assay CV is about 8%. We tested 30 haemophiliacs with FVIII:C between <1 IU/dL and 6 IU/dL as measured in the one-stage clotting assay using the FVIII-CAT assay. For genetic defects related to moderate haemophilia, the FVIII-CAT test finds FVIII levels that are in good agreement with those determined with the one-stage assay. Of the 21 haemophilic patients with FVIII <1 IU/dL, 4 patients exhibit a mild bleeding phenotype. When we applied TF-initiated thrombin generation, patients with mild clinical phenotype showed significantly higher endogenous thrombin potentials.

***Conclusion*:** The novel developed FVIII assay measures accurately FVIII levels below 1 IU/dL. Its application demonstrated that the clinical heterogeneity in individuals with less than 1 IU/dL FVIII is not associated with their FVIII level.

Introduction

Haemophilia A is a genetic disorder resulting from the deficiency of coagulation factor VIII (FVIII). In spite of undeniable improvements in the management of haemophilia A, there is still no validated laboratory test that predicts the individual bleeding phenotype. Neither is it known what threshold level of FVIII is required to stop bleeding.[1] Currently available FVIII assays are based upon the correction of the prolonged activated partial thromboplastin time (aPTT) of FVIII-deficient plasma by dilutions of test plasma (one-stage method) or by measuring the generation of prothrombinase activity by clotting time or chromogenic methods (two-stage method). The one-stage assay is widely used because of the simplicity of the method, but the coefficients of variation within laboratories is high (10-20%) and is incapable of measuring FVIII levels less than 1 IU/dL.[2, 3] The two-stage assay is more complex. Usually, FVIII dependent FXa formation is determined with a chromogenic substrate, but also this assay is unable to measure accurately FVIII levels below 1 IU/dL.[3, 4]

It is well established that there can be large discrepancies between one-stage and two stage methods, e.g. in approximately 40% of mild haemophilia A patients the one-stage FVIII level can be two-fold higher than the two-stage or chromogenic FVIII level.[5-8] Most intriguing is the observation that the level of FVIII, determined with the usual assays, does not reflect the clinical phenotype, for example 3-10% of patients with less than 1 IU/dL FVIII exhibit a milder bleeding phenotype. Indeed, the need for an accurate FVIII assay is generally appreciated.[9, 10]

It is increasingly being recognized that the transient thrombin generation profile in clotting plasma is a better indication of the function of the blood coagulation system than the clotting time.[9-11] McIntosh et al.[12] used a modification of a thrombin generation test developed by Macfarlane and Biggs[13] to correlate FVIII levels and clinical phenotype. However, this test is cumbersome, time-consuming and is probably not suited for the clinical practise by lacking a precise correlation between thrombogram parameters (e.g. endogenous thrombin potential (ETP), thrombin peak value) and FVIII activity. It is becoming increasingly apparent that the calibrated automated thrombin generation assay (CAT)[14], is a potential candidate to replace currently used assays in the management of haemophiliacs.[4, 15]

Here we report a modification of the CAT assay that enables an accurate measurement of FVIII plasma levels below 1 IU/dL. This assay might become a useful laboratory tool in managing haemophilia A.

Materials and Method

Reagents

Bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), thrombin calibrator, activated bovine Factor IX (FIXa; Synapse BV, Maastricht, the Netherlands), thrombin substrate Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC; Bachem, Bubendorf, Switzerland), recombinant tissue factor (TF; Innovin Dade Behring, Marburg, Germany), FVIII-deficient plasma (Haematologic Technologies, Vermont, USA), FV-deficient plasma (George King Biomedical, Overland Park, Kansas) and human plasma-derived FVIII (AAfact; Sanquin, Amsterdam the Netherlands) were obtained from the indicated vendors. Phospholipid (PL) vesicles contained 20% phosphatidylserine, 60% phosphatidylcholine and 20% phosphatidylethanolamine (Avanti, Alabama, USA). Corn trypsin inhibitor (CTI) was purified as described.[16] Functional TF concentration was determined with a FXa generation assay, where a fixed amount of FVIIa and FX were titrated with TF.

Patients

Thirty adult haemophilia A patients between 19 and 65 years of age (mean = 40.8) routinely treated in the Lyon Clinical Haemostasis Unit (France) were included, after obtaining written informed consent according to the Helsinki declaration. The study was approved by the Lyon University Hospital's ethical committee. Patients included in the present study did not receive primary prophylaxis. Patients presenting severe recurrent bleeds were later placed on secondary prophylaxis and were considered as severe bleeders. Therefore, prophylactic treatment will not lead to a misclassification of patients.

Of these haemophilia A patients, 21 were severe haemophiliacs (FVIII:C <1 IU/dL), 8 moderate (FVIII:C 1-5 IU/dL) and 1 mild (FVIII:C >5 IU/dL). Genetic defect linked to haemophilia was already identified in each patient and results were available in their clinical records. None of the patients had anti-FVIII inhibitors and they did not receive any FVIII infusion in the last 5 days preceding the tests. To establish their clinical bleeding phenotype, the clinical history of each patient was examined. Independently of their plasma FVIII activity, severe bleeders were defined as those presenting the combination of at least 2 of the following clinical indicators: i) patients on secondary prophylaxis after recurrent bleeding episodes, ii) patients with a history of frequent spontaneous bleeding episodes (>30 episodes) and iii) patients presenting severe

radiological haemophilic arthropathy with a Petterson score at 3-4. Mild bleeding was defined as bleeding phenotype that does not meet the criteria for severe clinical bleeding phenotype.

Plasma preparations

Normal pooled plasma (NPP): Blood samples from healthy donors (n = 8) were collected by venipuncture using a 18 G needle, discarding the first 2-3 mL, and allowing the blood to drip freely into open tubes containing 1/10 volume of trisodium citrate (129 mM). For the preparation of platelet poor plasma, whole blood samples were centrifuged twice at 2630 x g for 10 min. Immediately after centrifugation, plasma samples were pooled together, snap-frozen in liquid nitrogen and stored at -80°C. The resulting pool contained 91 IU/dL FVIII as measured by one stage aPTT-based clotting assay.

Haemophiliac plasma: Blood was taken from patients through a 21-G needle with a light tourniquet into S-Monovette® tubes (Sarstedt, Orsay, France) containing 0.106 mol/L trisodium citrate (1:9, volume:volume) and loaded with corn trypsin inhibitor (Haematologic Technologies Inc., Vermont, USA) at a concentration of 18.3 µg/mL in whole blood. Platelet-poor plasma (PPP) was obtained by double centrifugation at 2500 × g for 15 min at room temperature.

Thrombin generation assay

The calibrated automated thrombogram (CAT) assay was performed as previously described.[14] Briefly, 80 µL test plasma and 20 µL trigger reagent (TF or FIXa and 4 µM phospholipids) were mixed in 96-well round-bottom microtiter plates (Immunulon2HB, ThermoLab System, Helsinki, Finland). The plate was pre-warmed for 10 min at 37°C. Thrombin generation was started by the addition of 20 µL Z-GGR-AMC (2.5 mM) and $CaCl_2$ (100 mM). The calibration was performed with a mixture of plasma, thrombin calibrator (final thrombin activity of 100 nM) and ZGGR-AMC. From the thrombogram the following parameters were calculated: lagtime (min), ETP (nM.min; area under the thrombin curve corrected for the $\alpha_2$macroglobulin-thrombin activity), thrombin peak height (nM) and velocity index (VI, nM/min equals thrombin peak value/(time to peak - lagtime)).

Determination of FVIII activity in suspensions of activated platelets

Washed platelet suspensions were isolated and activated as recently described.[17] Briefly, washed platelets ($6x10^8$ platelets/mL) in platelet buffer (10 mM HEPES [N-2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid], 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, pH 7.5, containing 2 mg/mL bovine serum albumin and 2 mg/mL glucose) were incubated with 100 µM PAR-1 activation peptide SFLLRN (Bachem, Bubendorf, Switserland) and 3 mM $CaCl_2$ for 10 min at 37˚C. The platelet suspension was centrifuged (2 min at 2300 x g) and the supernatant was diluted 4-fold with FVIII-deficient plasma or FV-deficient plasma and assayed for FVIII activity and FV activity, respectively, using the FIXa-driven TG assay in the presence of 4 µM phospholipid vesicles.

Data analysis

Data analysis was performed using dedicated software (Thrombinoscope BV, Maastricht, the Netherlands). The thrombograms shown are the mean of a set of three separate measurements. The thrombin generation parameters were calculated from the separate curves and shown as the mean ± SD. Spearman and Mann-Whitney tests were used for statistical analysis.

Results

Dose-response of TG to TF and FIXa in normal and haemophiliac plasma.

We addressed the question which trigger of blood coagulation, TF or FIXa, and which parameters could be useful for an accurate FVIII-CAT assay in platelet poor plasma. NPP and FVIII-deficient plasma were activated with varying trigger concentrations and TG was measured in the presence of CTI (10 µg/mL). No thrombin could be detected in the absence of TF or FIXa (data not shown). As shown in Figure 1A, TF dose-dependently increased TG in FVIII-deficient plasma (Figure 1A). Compared with TF-initiated TG in NPP (Figure 1B), the thrombin peak values were lower, indicating that the TF-triggered TG is only partially dependent on the FVIII-dependent TG pathway. No TG was observed in FIXa-activated FVIII-deficient plasma (data not shown). Increasing concentrations of FIXa did increase thrombin peak values and decreased the lag time in NPP (Figure 1C). We established that FIXa in plasma retained its activity during the assay-time: a pre-incubation of FIXa (1 nM) in plasma for 30 min at 37°C did not affect

FIXa-driven TG. The thrombogram parameters did not vary significantly, e.g. thrombin peak height at time zero was 436 ± 12 nM (mean ± SD, n = 3) and after 30 minutes 421 ± 12 nM (mean ± SD, n = 3). This observation confirms an earlier finding of a very slow inactivation of plasma FIXa in the absence of heparin.[18]

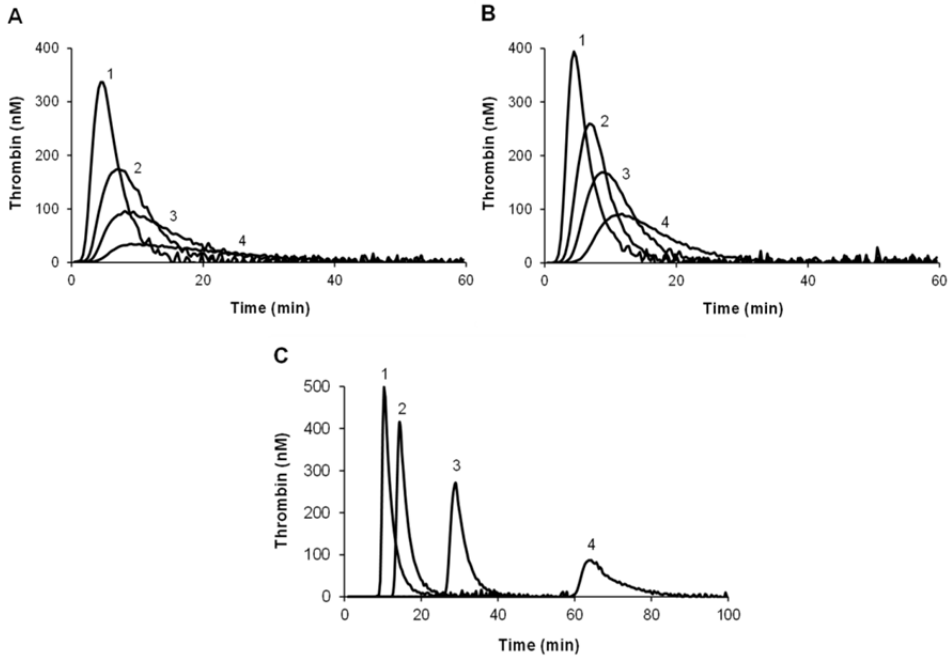

Figure 1: Trigger dependency. (A, B) Thrombograms of TF (1: 5 pM; 2: 2 pM; 3: 1 pM; and 4: 0.5 pM TF) triggered FVIII-deficient plasma (A) and normal plasma (B). (C) Thrombograms of FIXa (1: 5 nM; 2: 1 nM; 3: 0.4 nM; and 4: 0,2 nM) triggered normal plasma. All experiments were performed in the presence of CTI (10 µg/mL) and phospholipids (4 µM).

The thrombogram parameters ETP and thrombin peak height as a function of the TF and FIXa concentration are depicted in Figure 2. The data suggest that the lowest detection limit of FVIII activity levels in haemophilic plasma will be obtained with FIXa and not with TF. Even at low TF concentrations the FVIII dependency, as reflected by differences in both ETP and thrombin peak height (Figure 2A and 2B), is much smaller compared to triggering with FIXa (Figure 2C and 2D).

The precision of the TG parameters at the different FIXa concentrations was determined and revealed that the most consistent data were obtained with saturating amounts of FIXa (> 2 nM). In addition, at these concentrations addition of CTI had no

effect on TG parameters: in the absence and presence of CTI (10 µg/mL), thrombin peak values were 513.3 ± 4.3 nM (mean ± SD, n = 3) and 513.1 ± 13 nM (mean ± SD, n = 3), respectively.

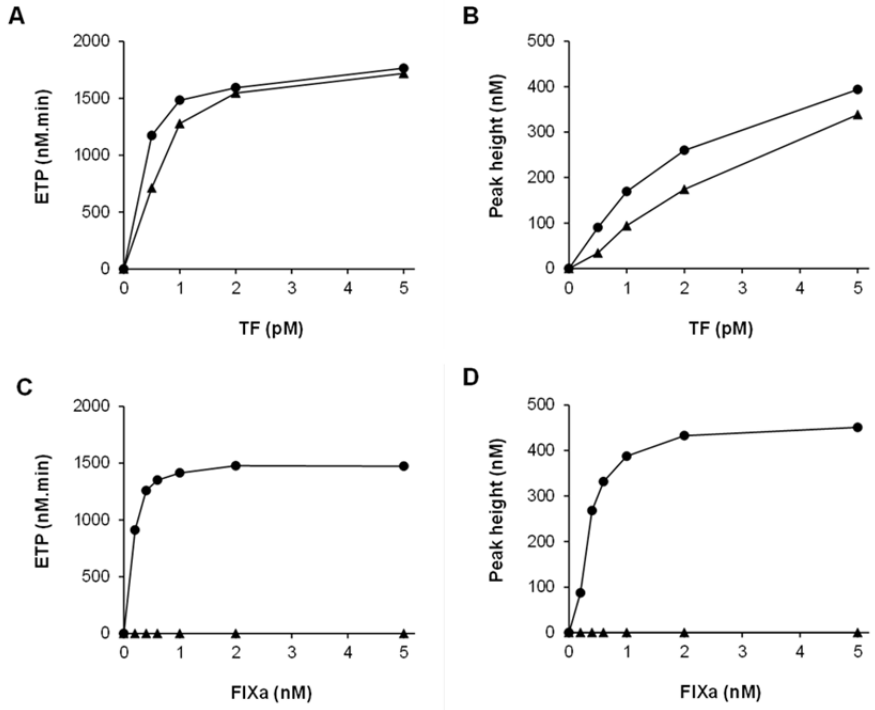

Figure 2: TG parameters. (A, B, C, D) The TG parameters were determined from the thrombograms of which the means are shown in Figure 1. NPP (●) and FVIII-depleted plasma (▲) were activated with TF (A, B) or FIXa (C, D).

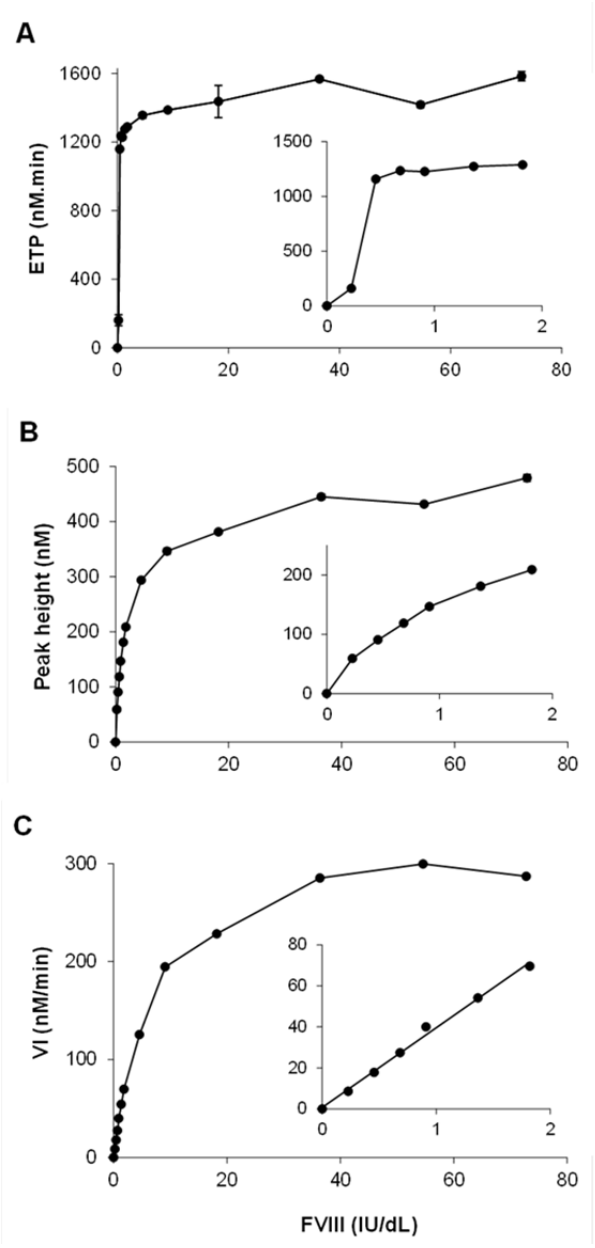

Figure 3: Dependency of TG parameters on plasma FVIII activity. NPP was diluted with FVIII deficient plasma to the indicated FVIII activities and activated with 5 nM FIXa and phospholipids (4 μM). Insets show plots of thrombogram parameters versus plasma FVIII levels between 0 and 2 IU/dL.

Dose-response of TG to FVIII in normal plasma

NPP was diluted with FVIII-deficient plasma to obtain plasma samples that varied between 0 and 80 IU/dL FVIII. Plasma samples were activated in the absence of CTI with 5 nM FIXa and 4 μM PL. The TG parameters ETP, thrombin peak height and VI are shown in Figure 3. All TG parameters showed saturation at FVIII levels higher than 40 IU/dL. ETP reached saturation already at about 10 IU/dL (Figure 3A). As seen in the insets of Figure 3, only VI (regression coefficient (r) = 0.9945) showed a linear relationship with plasma FVIII levels up to 2 IU/dL. Consequently, this parameter was chosen to construct reference lines.

Performance of the FVIII-CAT assay

The intra-assay variability was assessed by performing the assay repeatedly (10 measurements) with FVIII-deficient plasma spiked with 0.5 IU/dL or 1 IU/dL recombinant FVIII. The coefficient of variation (CV) of VI was 2.5% and 3.1%, respectively. The CV of the thrombin peak values was 1.2% (0.5 IU/dL FVIII) and 1.4% (1 IU/dL FVIII). The day-to-day variability was assessed with the reference materials 0.5 and 1 IU/dL FVIII over a 4-month period on 15 occasions in triplicate. The CV of the velocity index was 7% (0.5 IU/dL FVIII) and 10% (1 IU/dL FVIII). CV values for thrombin peak heights were 12% (0.5 IU/dL FVIII) and 13% (1 IU/dL FVIII).

The lower limit of detection of the assay (0.05 IU/dL) was determined from the y-intercept of the VI-FVIII reference line. The y-intercept was 0.9 ± 0.4 nM/min (mean ± SD, n = 3). It is apparent that the lowest detection limit is obtained with FVIII-deficient plasma that does not contain FVIII activity. Figure 4 shows a few examples of commercial available plasmas that cannot accomplish this requirement. FVIII-deficient plasma A, used throughout this study, contained virtually no detectable FVIII activity (VI=0). FVIII-deficient plasma B contained 0.1 IU/dL FVIII and immuno-depleted FVIII-deficient plasma C did not contain FVIII activity, but this plasma neutralized about 0.5 IU/dL FVIII. All FVIII deficient plasmas were immuno-depleted and contained VWF.

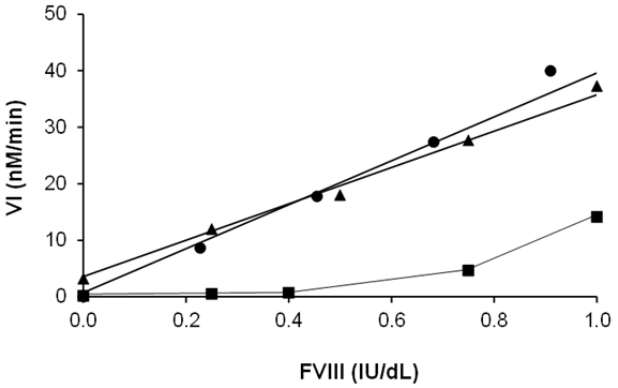

Figure 4: Assay accuracy and FVIII-deficient plasma. Reference curves were prepared by diluting NPP in FVIII-depleted plasma A (●), FVIII-depleted plasma B (▲) and FVIII-depleted plasma C (■). Velocity index is plotted versus the FVIII activity.

Platelet FVIII

Because of the precision and low detection limit of the FVIII-CAT assay, we investigated the notion that platelets have undetectable FVIII levels.[19] To this end, platelets were isolated from blood of a healthy volunteer and activated with the PAR-1 agonist SFLLRN. Subsequently, FVIII activity was measured in the supernatant of the suspension of activated platelets with the FVIII-CAT assay. As a positive control the lysates were also assayed for FV activity. The thrombograms, shown in Figure 5, clearly indicate that platelet activation does release FV activity but no FVIII activity.

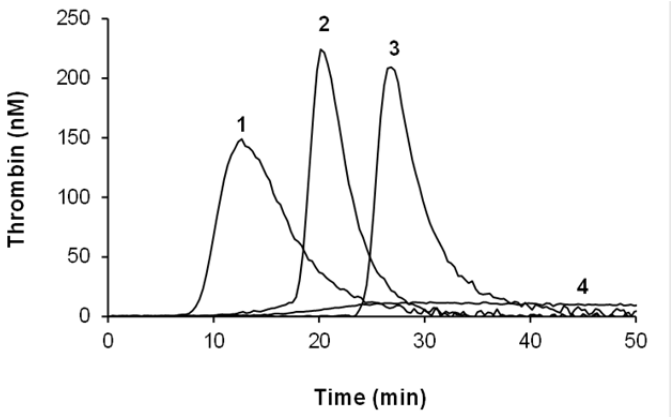

Figure 5: Platelet FVIII. Platelets ($6x10^8$/mL) were activated with 100 µM TRAP-6 and the supernatant measured for FVIII (curve 4) and FV activity (curve 3) using FVIII- and FV-CAT assay. Thrombograms of FV-deficient plasma spiked with 1 IU/dL normal plasma (curve 2) and FVIII-deficient plasma spiked with 1 IU/dL normal plasma (curve 1) are included.

Table 1: FVIII determination with FVIII-CAT assay and ETP of TF-activated plasmas of haemophilia A patients.

Patient	FVIII CAT-assay IU/dL	FVIII One stage IU/dL	FVIII Mutation	Clinical bleeding phenotype	ETP* nM.min
1	<0.05	<1	Intron 22 inversion	Severe	393
2	<0.05	<1	Intron 22 inversion	Severe	491
3	<0.05	<1	Intron 22 inversion	Severe	291
4	<0.05	<1	Intron 22 inversion	Severe	509
5	<0.05	<1	Intron 22 inversion	Severe	358
6	<0.05	<1	Intron 22 inversion	Severe	547
7	<0.05	<1	Intron 22 inversion	Severe	52
8	<0.05	1	Intron 22 inversion	Severe	426
9	<0.05	<1	Intron 22 inversion	Mild	907
10	<0.05	<1	Intron 22 inversion	Mild	867
11	<0.05	<1	Intron 1 inversion	Severe	510
12	<0.05	<1	3 bp Deletion 2014-2017	Severe	313
13	<0.05	<1	Insertion A 4484 and deletion C 4486-4487	Severe	208
14	<0.05	<1	Deletion G 1427 and stop codon at 1445	Mild	874
15	0.40	<1	22 bp Deletion 2067-2088	Severe	544
16	<0.05	<1	No mutation detected	Severe	559
17	<0.05	<1	Thr 118 Ile	Mild	356
18	<0.05	<1	Thr 118 Ile	Severe	506
19	0.38	<1	Thr 118 Ile	Severe	562
20	0.12	<1	Thr 118 Ile	Severe	426
21	0.64	<1	Thr 118 Ile	Severe	387
22	1.95	1	No mutation detected	Severe	134
23	1.13	1	Ser 260 Cys	Severe	463
24	3.27	2	Leu 566 Phe	Mild	700
25	3.37	2	Ser 241 Cys	Severe	575
26	5.80	4	Leu 523 Leu	Mild	844
27	3.73	4	No mutation detected	Severe	756
28	2.88	5	Leu 431 Phe	Mild	963
29	3.57	5	Ile 2197 Ser	Mild	647
30	7.10	6	Gly 479 Arg	Mild	928

bp: base pair; *ETP was calculated from thrombin generation measurements in plasma activated with 1 pM TF.

Measuring FVIII in plasma of patients with haemophilia A

Table 1 depicts the FVIII levels in undiluted plasma measured with the FVIII-CAT assay and plasma FVIII levels measured by the one-stage assay. The genetic defects linked to haemophilia A and the clinical bleeding phenotype of the patients are also included. For patients with moderate (1-5 IU/dL FVIII:C) and mild haemophilia A (>5 IU/dL FVIII:C), FVIII-CAT results were significantly correlated to the one-stage FVIII measurements ($r = 0.71$; $P = 0.03$).

Of the 21 haemophilia A patients with FVIII:C <1 IU/dL), 4 patients had FVIII-CAT levels between 0.12 and 0.64 IU/dL and 17 patients had no detectable FVIII (< 0.05 IU/dL). The data also indicate that 4 patients showed a clinical mild bleeding tendency, in spite of the absence of detectable FVIII activity.

The plasmas of all patients (n = 30) were activated with 1 pM TF and TG was measured. ETP values are shown in Table 1. The average ETP value of the patients with severe clinical bleeding is 448 ± 142 nM.min (mean ± SD, n = 21) and 787 ± 192 nM.min for patients with a mild bleeding tendency (mean ± SD, n = 9). Thus, independent of FVIII levels, mild bleeding is associated with a significant ($P < 0.001$) higher ETP value. For the group of patients with no detectable FVIII activity the ETP values in case of severe bleeders and mild bleeders are 426 ± 113 nM.min (mean ± SD, n = 17) and 751 ± 264 nM.min (mean ± SD, n = 4), respectively ($P = 0.052$).

Discussion

There appears to be a consensus that there is a need for new assays to monitor haemophilia A diagnosis and treatment.[10, 20-22] In this paper we describe the development and performance of a calibrated and automated FVIII activity assay that complies with the need of measuring FVIII activity in the range of 0 – 2 IU/dL with high precision.

The data show that even at low TF concentrations, TG proceeds at least partly via the extrinsic pathway and thus independent of FVIII. None of the TF-dependent thrombogram parameters (e.g. ETP and thrombin peak height) showed a reliable correlation with plasma FVIII levels. In contrast, TG triggered with FIXa (5 nM) is explicitly dependent on the presence of FVIII. At low FVIII levels, the thrombogram parameters ETP, thrombin peak, VI and time-to-peak varied with the FVIII activity. Maximal ETP values were readily obtained with FVIII >0.5 IU/dL. For thrombin peak

values the saturating amount of FVIII was >30 IU/dL. Because VI values increased linearly in the required range (0-2 IU/dL), only this parameter appeared to be useful for our FVIII measurements. The FVIII-CAT assay can be performed with high precision (intra-assay variation of 2.5% and inter-assay variation of 7%) and has a low detection limit (0.05 IU/dL).

A laboratory assay that can detect individuals at lower bleeding risk would be of interest to individually tailor prophylaxis in patients with severe haemophilia. Of severe haemophilia A patients, 10 to 15% exhibit a milder clinical bleeding phenotype. The question of "do severe haemophilia patients with higher plasma FVIII levels close to 1 IU/dL present lower risk of bleeding than those with FVIII lower than 0.5 IU/dL?" is still open to debate because routine FVIII measurements methods are not accurately enough at FVIII concentrations below 1 IU/dL. Our FVIII measurements found no correlation between plasma FVIII levels less than 1 IU/dL and the clinical severity of bleeding symptoms. Of the 21 haemophilic patients with FVIII levels lower than 0.05 IU/dL, 4 patients exhibit a mild bleeding phenotype. It is unlikely that the inter-individual variability of bleeding phenotype in patients with severe haemophilia is attributed to differences in functional amounts of platelet FVIII for the obvious reason that platelets do not release or are void of FVIII activity. It has been suggested that haemophilia A patients with no FVIII activity may be rescued from severe bleeding because of their decreased levels of plasma anticoagulants like tissue factor pathway inhibitor, protein S or antithrombin.[23-25]

In conclusion, we found that that the bleeding phenotype in haemophilia A patients is not related with FVIII levels in the range of 0-1 IU/dL. We used a TF-triggered thrombin generation assay to confirm that within the group of patients with < 1 IU/dL FVIII, patients might be rescued from severe bleedings by factors that increase thrombin generation. It is thus obvious that because of enhanced procoagulant mechanisms, the FIXa-triggered FVIII-CAT assay cannot be used to assess bleeding risk. It remains to be demonstrated in a multicenter study with a large number of mild bleeders whether TF-triggered thrombin generation assays are useful to predict the bleeding tendency in an individual and to detect other thrombin generation enhancing factors that might be associated with the clinical phenotype.

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Chapter 6

Conformation of beta2glycoprotein I and its effect on coagulation

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Abstract

$\beta_2$glycoprotein I (β_2GPI) is the major antigen in the antiphospholipid syndrome. It has been shown that β_2GPI can adapt to different conformations, a circular, a S-shaped and a J-shaped conformation. In literature anticoagulant properties of β_2GPI have been indicated, though there is no consensus on how β_2GPI exerts a certain action. This article will first review existing data on the conformation of β_2GPI. In addition, we will investigate whether the conformation of β_2GPI plays a role in in the proposed anticoagulant activity of β_2GPI. We investigated the effect of native β_2GPI and phospholipid-bound β_2GPI on thrombin generation (TG). Native β_2GPI was found to have no significant effect on the TG regardless of the concentration of tissue factor. On the contrary, β_2GPI preincubated with phospholipids significantly inhibited TG triggered with low TF concentration, suggesting an effect on the intrinsic pathway. This indicates that native β_2GPI in circulation obtains its anticoagulant activity in the presence of anionic phospholipids such as activated blood cells thereby serving as an inhibitory modulator in hemostasis.

Introduction

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by vascular thrombosis and obstetrical complications, and serologically by the presence of antiphospholipid antibodies.[1] Other clinical symptoms that can be prominently present are kidney-faillure, livedo reticularis and neurological signs, though these have not been accepted as official criteria due to specificity reasons.[2] Antiphospholipid antibodies can be detected via three different assays as described by the official revised criteria for APS diagnosis; anti-$\beta_2$glycoprotein I (β_2GPI) antibody assays, anticardiolipin antibody assays and prolongation of phospholipid-dependent coagulation assays, preferably dRVVT-or-APTT based assays.[3, 4] The major antigen of these so-called antiphospholipid antibodies has been found to be β_2GPI.[5] β_2GPI is an evolutionary well conserved, highly abundant 48 kDa protein consisting of five short consensus repeat domains connected by 3 or 4 amino acids that form a flexible linker between the domains.[5] The fifth domain includes a large positively charged patch harboring the binding site for anionic phospolipids.[6] Domain I contains epitope glycine 40- arginine 43 recognized by the subpopulation of autoantibodies that correlate best with thrombotic manifestations of APS.[7-9] Interestingly, several years ago various studies showed that β_2GPI harbours anticoagulant activity by inhibiting factor (F) XII(a) initiated coagulation.[10-12] However, other studies showed contradicting results and so far no consensus has been reached.[13, 14] The last few years several studies have been published describing different conformations of β_2GPI.[15-18] Therefore we decided to investigate whether the conformation of β_2GPI has an influence on the proposed anticoagulant properties of β_2GPI. In this study we will first summarize the previously published data regarding the conformation of β_2GPI, followed by recent data produced in our laboratory in order to study the role of β_2GPI in coagulation in relation to its conformation.

Different conformations of β_2GPI

β_2GPI is a protein discovered in 1961[19-20], consisting of five short consensus repeats, also referred as sushi domains.[21] Domains I, II, III and IV are approximately 60 amino acids in size. Domains III and IV contain attachment points for four carbohydrate chains making up 20% w/w of the total molecular mass of the protein. Domain V is different

from the other four domains and consists of 82 amino acids including a C-terminal hydrophilic positively charged patch enabling β_2GPI to bind anionic surfaces such as activated blood cells.[15] In 1999, 2 independent groups published the crystal structure of β_2GPI.[14, 15] Both studies have established β_2GPI as a J-shaped protein with domain I pointing vertically upward from the surface and domain V with the positive patch projected downward. The upward positioning of domain I is in line with our previous reports in which we have shown that domain I is the main domain involved in binding of thrombosis-related antiphospholipid antibodies. In the study of Schwarzenbacher et al. domain V contained a hydrophilic loop, which is thought to be injected into the phospholipid membrane.[15] In the study of Bouma et al. this loop was proven to contain two loose ends indicating that this crystalized form of β_2GPI was cleaved at position 316–317.[14] Cleaving of β_2GPI at these amino acids results in loss of the phospholipid-binding capacity of the protein.[22]

Although both groups have established the same structure using crystalization as optic tool, β_2GPI has been suggested to adopt other conformations. A hint towards different conformations was the failure of isolation of antigen-antibody complexes from the blood of patients containing anti-β_2GPI antibodies. In 2002, Hammel et al. have shown by small angle X-ray scattering experiments that β_2GPI in solution is not J- but S-shaped [16]. Interestingly enough, in this model epitope G40-R43 on domain I was covered by a carbohydrate chain. We have shown that this hidden epitope G40-R43 on domain I is responsible for the binding of thrombosis-related antiphospholipid antibodies.[23] Apparently β_2GPI in solution is present in its native S-shaped conformation with epitope G40-R43 covered by a carbohydrate chain, but can adapt to a J-shaped conformation upon binding to an anionic surface. Further proof for this hypothesis was given by the fact that upon cleaving off the carbohydrate chains, anti-β_2GPI antibodies do interact with “native” β_2GPI.[23]

Recently a new chapter has been added to this story. Agar and colleagues have published a study in which native β_2GPI was found to adopt a circular conformation.[17] In this study electron microscopy was used to visualize β_2GPI on a special grid. Whether the grid has any influence on the conformation of β_2GPI is not known. As with the previous study of Hammel et al.[16], in this study epitope G40-R43 was also shown to be hidden, this time not by a carbohydrate chain but by domain V. Although the resolution did not reveal its exact contact surface, it seems that domain V with its C-terminal positive patch was positioned between domain I and II. Upon binding to negatively charged surfaces (with its positively charged domain V), the conformation

of β_2GPI is disturbed.[17] The closed conformation of β_2GPI opens up, exposing the cryptic site within domain I on the outside of the protein. Recently, another study has indicated that not only binding to anionic phospholipids or to an antibody "opens up" β_2GPI, but also lipopolysaccharides derived from Escherichia Coli were found to transform β_2GPI from a circular to a J-shaped conformation.[24] At this moment it is not known whether β_2GPI can adapt to either a circular, S-shaped or J-shaped conformation or that all conformations exist and some serve as intermediates.

Influence of different conformations of β2GPI on TG

Given the contradicting results of β_2GPI on coagulation and the discovery of different conformations of β_2GPI, we investigated the influence of both the native plasma conformation of β_2GPI as well as β_2GPI bound to phospholipids (which is regarded as the open activated conformation of β_2GPI) on thrombin generation (TG). Indeed, upon pre-incubation with anionic surfaces such as phospholipids, β_2GPI undergoes a major conformational change from its native closed, either circular or S-shaped, to an open J-shaped conformation, resulting in the exposure of a cryptic epitope in domain I.[17, 25] Thrombin activity was calculated as a function of time by comparing the fluorescent signal from the thrombin-generating sample to that from a known stable concentration of thrombin activity measured simultaneously in a parallel sample, as described previously.[26]

In the first set of experiments, TG was triggered in normal pooled plasma (NPP) with different concentrations of recombinant human tissue factor (TF), ranging from 0.5-5 pM, in the presence of 4 μM phospholipids, containing 20 mol% phosphatidylserine, 60 mol% phosphatidylcholine and 20 mol% phosphatidylethanolamine. Experiments were carried out in the absence or presence of plasma-derived β2GPI. This native β2GPI (25 μg/ml) did not influence the TG initiated with 0.5 pM TF, not even at a higher concentration (50 μg/ml) (Figure 1A).

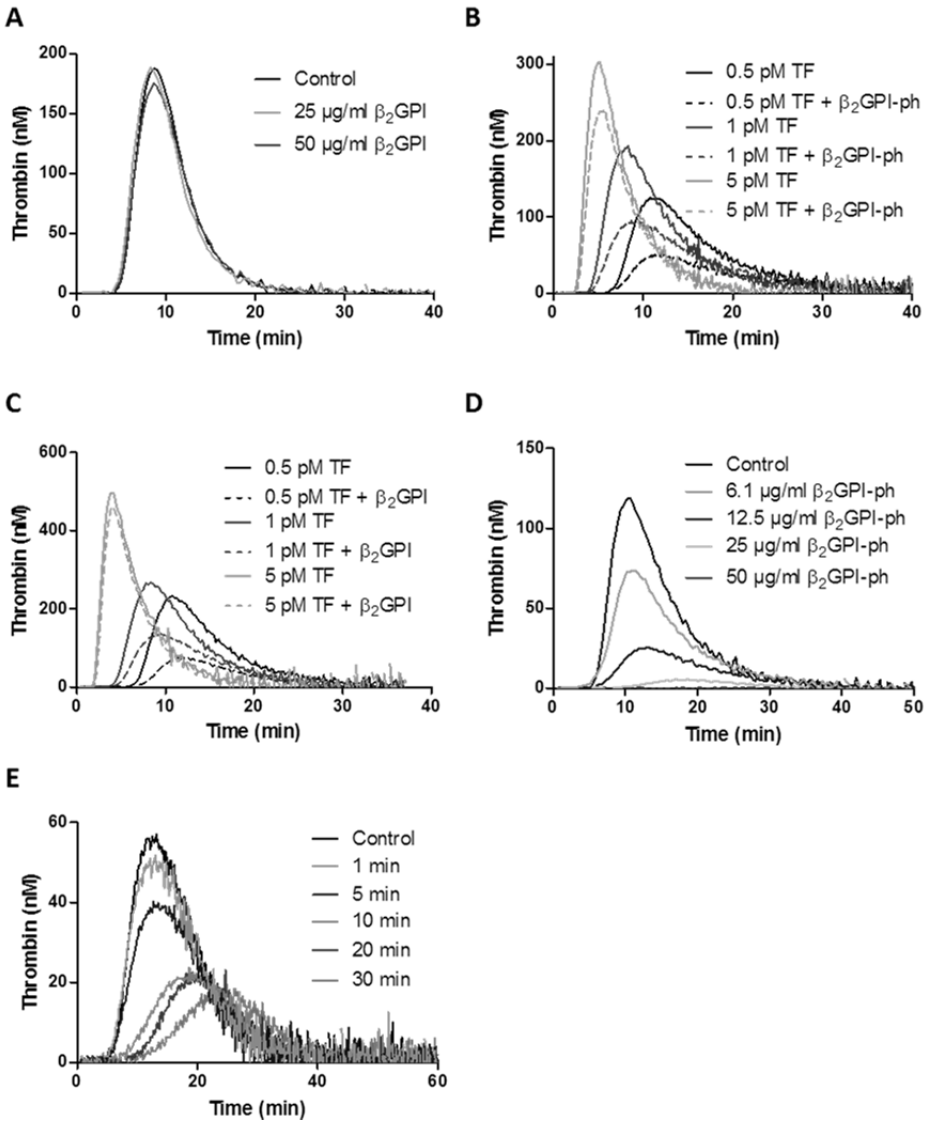

Figure 1: Open β_2GPI dose-dependently inhibits the thrombin generation of normal pooled plasma (NPP) triggered with low concentrations of TF. A. Thrombin generation was triggered in NPP with 0.5 pM TF and 4 µM phospholipids, in the absence or presence of plasma-derived β_2GPI in the indicated concentrations. B-C. Thrombin generation was triggered in NPP with different concentrations of TF (0.5-1-5 pM) and 4 µM phospholipids, in the absence or presence of open activated β_2GPI (25 µg/ml). Activation of β_2GPI was achieved by pre-incubation with phospholipids (48 µM) for 15 minutes (B) or by coating of the β_2GPI on an anionic ELISA plate (C). D. Different concentrations of β_2GPI (6.1 to 50 µg/ml) in its open conformation (induced by pre-incubation with phospholipids for 15 minutes) were added. E. The effect of the pre-incubation time with phospholipids, varying from 0–30 minutes, on the anticoagulant effect of β_2GPI is shown. A-E. Thrombin generation experiments were run in triplicate.

To study the effect of β2GPI in its open conformation, β2GPI was pre-incubated with 48 µM phospholipids (β2GPI–ph) for 15 minutes before being tested in the TG assay. In the presence of 5 pM TF, β2GPI–ph (25 µg/ml) did not significantly influence the TG (Figure 1B; peak (control) = 302.6 nM versus peak (β2GPI) = 241.2 nM). However, β2GPI–ph (25 µg/ml) decreased the TG initiated by low TF concentrations (0.5-1 pM), as illustrated by a reduced peak height (Figure 1B; peak (0.5 pM TF, control) = 124.4 nM versus peak (0.5 pM TF, β2GPI) = 51.4 nM; peak (1 pM TF, control) = 192.5 nM versus peak (1 pM TF, β2GPI) = 92.4 nM). Alternatively, to obtain β2GPI in its open J-shaped conformation, β2GPI was coated on an anionic ELISA plate. Similar as to pre-incubation with β2GPI pre-incubated with phospholipids, the coated β2GPI decreased the TG initiated by low TF concentrations (0.5-1 pM) but not by 5 pM TF (Figure 1C; peak (0.5 pM TF, control) = 231.9 nM versus peak (0.5 pM TF, β2GPI) = 73.9 nM; peak (1 pM TF, control) = 266.7 nM versus peak (1 pM TF, β2GPI) = 134.2 nM; peak (5 pM TF, control) = 497.3 nM versus peak (5 pM TF, β2GPI) = 460.7 nM).
Subsequently, the dose-dependency of this anti-coagulant effect of β2GPI–ph, as well as the effect of the pre-incubation time with phospholipids was investigated. TG was initiated with 0.5 pM TF. As illustrated in Figure 1D, the anti-coagulant effect of β2GPI pre-incubated with phospholipids already appeared at a concentration of 6.1 µg/ml, and a concentration of 50 µg/ml completely abolished the TG. As to the effect of the incubation period, increasing incubation times resulted in a further decrease in peak height and increase in lag time (Figure 1E).

Discussion

Earlier studies investigating the role of β_2GPI in hemostasis have found contradicting results, regarding the anti- or procoagulant property of β_2GPI. Recently, more insight into the dynamic conformation was found. β_2GPI seems to be able to adopt different conformations depending on its interaction with an anionic surface; an S-shaped, a circular and a J-shaped conformation. We studied these new tie-ins in the conformation of β_2GPI in relation to a possible effect on coagulation, measured by TG. Indeed, we have found that β_2GPI in its native conformation does not affect any of the parameters of TG. Preincubation with phospholipids, thereby inducing a conformational change from its native to an open J-shaped conformation, did reveal anticoagulant properties for β_2GPI on TG. Altogether our data suggest the following

working hypothesis: β_2GPI in circulation in its native conformation (either S-shaped or circular) is unable to exert its anticoagulant activity as apparently the activity site is hidden. At the site of vascular damage β_2GPI binds to activated cellular surfaces resulting in a J-shaped conformation. The active site, suggesting to be an epitope on domain I, is no longer covered resulting in the inhibition of coagulation. In this hypothesis β_2GPI serves as a modulator of coagulation. A role for domain I as modulator in coagulation is an appealing hypothesis, especially when realizing that domain I harbours the epitope G40-R43 responsible for binding to thrombosis-related antibodies. These antibodies might interfere with the anticoagulant properties thereby causing thrombosis. Future studies need to be done to study whether this is likely to happen in patients suffering from the antiphospholipid syndrome.

Interestingly enough it seems that for both proposed native conformations, S-shaped and circular β_2GPI, carbohydrate chains play a role.[16, 17] As mentioned before, epitope G40-R43 was shown to be covered by a carbohydrate chain and upon binding to an anionic surface this epitope on domain I was accessible for antiphospholipid antibodies to bind. In the circular conformation the C-terminal part of domain V is interacting with the intersection between domain I and II. Both surfaces are positively charged, indicating that, based on those charges, they would push each other away. It is likely that the negatively charged carbohydrate chain, that is positioned on top of the domain I-II intersection, serves as glue between domain V and domains I-II.

β_2GPI is an evolutionary conserved protein, quite abundantly present in many species. Additionally, β_2GPI is well conserved between species.[27] If the charges in β_2GPI are important for its function, both the interaction sites on domain I-II and domain V should be quite well conserved. And indeed, looking at Figure 2, it can be observed that in domain I and II the intersection is best conserved, with arginine 43 present in all species. In domain V it was already shown that the KNEKK site was conserved but also other lysines show extremely good conservation. This evolutionary conservation indicates that both sites in β_2GPI have important roles. The positive charge in domain V is known to be crucial for phospholipid binding. The conservation in domain I suggest a role in the physiological activity of β_2GPI. Domain I of β_2GPI was shown to bind thrombosis-related antibodies thereby indicating that this well conserved region has anticoagulant properties.

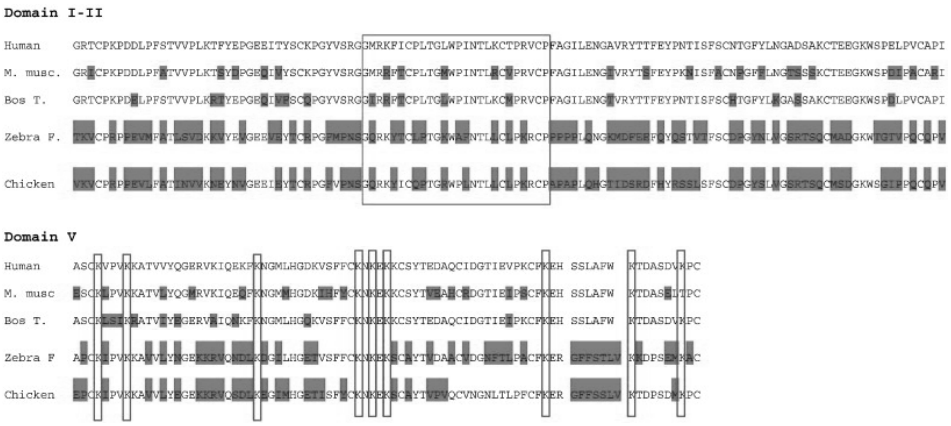

Figure 2: The interaction sites on domain I-II and domain V of β2GPI are quite well conserved between different species. In domain I and II the intersection is best conserved, with arginine 43 present in all species. In domain V both the KNEKK site and other lysines show extremely good conservation. This evolutionary conservation suggests that both sites in β2GPI have important roles in the function of the protein.

In conclusion, the newly generated data on the different conformations of β_2GPI to which it can adapt has opened new doors to study the physiological role of β_2GPI in hemostasis. Furthermore it might also help to further characterize the prothrombotic effects of antiphospholipid antibodies. We have found *in vitro* proof for β_2GPI to be involved as anticoagulant agent, only when bound to phospholipids indicating a major role for the conformation of β_2GPI. Future studies are needed to elucidate the exact role of β_2GPI in hemostasis.

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Chapter 7

Additive roles of platelets and fibrinogen in whole-blood fibrin clot formation upon dilution as assessed by thromboelastometry

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Abstract

Background and aim: Blood dilution after transfusion fluids leads to diminished coagulant activity monitored by rotational thromboelastometry, assessing elastic fibrin clot formation, or by thrombin generation testing. We aimed to determine the contributions of blood cells (platelets, red blood cells) and plasma factors (fibrinogen, prothrombin complex concentrate) to fibrin clot formation under conditions of hemodilution *in vitro* or *in vivo*.

Methods: Whole blood or plasma diluted *in vitro* was supplemented with platelets, red cells, fibrinogen or prothrombin complex concentrate (PCC). Thromboelastometry was measured in whole blood as well as plasma; thrombin generation was determined in parallel. Similar tests were performed with blood from 48 patients, obtained before and after massive fluid infusion during cardiothoracic surgery.

Results: Addition of platelets or fibrinogen, in additive and independent ways, reversed the impaired fibrin clot formation (thromboelastometry) in diluted whole blood. In contrast, supplementation of red blood cells or prothrombin complex concentrate was ineffective. Platelets and fibrinogen independently restored clot formation in diluted plasma, resulting in thromboelastometry curves approaching those in whole blood. In whole-blood from patients undergoing dilution during surgery, elastic clot formation was determined by both the platelet count and the fibrinogen level. Thrombin generation in diluted (patient) plasma was not changed by fibrinogen, but improved markedly by prothrombin complex concentrate.

Conclusions: In dilutional coagulopathy, platelets and fibrinogen, but not red blood cells or vitamin K-dependent coagulation factors, independently determine thromboelastometry parameters measured in whole-blood and plasma. Clinical decisions for transfusion based on thromboelastometry should take into account the platelet concentration.

Introduction

It is still a matter of debate how insufficiencies of the hemostatic system lead to bleeding under conditions of massive fluid infusion during cardiothoracic surgery. Fluid infusion diminishes a variety of processes, including platelet activity, coagulation factor levels, thrombin generation and fibrin clot formation. The latter process is monitored by rotational thromboelastometry or thromboelastography, which are frequently used to monitor patients during surgery.[1-3] Elastic fibrin clot formation can be measured in whole blood samples, thus providing a rapid point-of-care method for goal-directed coagulation management of surgery or trauma patients with, for example, fibrinogen concentrate.[4]

Recently, we have shown that combined plasma measurement of thrombin generation and fibrin clot formation results in a better prediction of the bleeding risk after major surgery than the use of either test alone.[5] The rationale is that thrombin generation monitors the rate and extent of formed thrombin, as a central controlling enzyme of the coagulation cascade.[6] On the other hand, thromboelastometry, being more sensitive for fibrinogen[7, 8] and platelets[8, 9], reports on the more advanced stage of elastic fibrin clot formation. In patients with bleeding during major surgery or experiencing trauma, impaired hemostasis can be restored by administration of fibrinogen[10-12] or vitamin K-dependent coagulation factors (prothrombin complex concentrate, PCC).[10, 13, 14] Both types of concentrates are also effective in large animal models of injury-induced bleeding[15-17], suggesting that normalization of part of the coagulation factors helps to stop bleeding after dilution.

Vitamin K-dependent coagulation factors, platelets and fibrinogen all contribute to the process of elastic clot formation by providing enzymatic activity, strength and mass of a clot, respectively. However, it is still unresolved how, under conditions of dilutional coagulopathy, reduced levels of these blood components interact to limit fibrin clot formation, nor is it clear how red blood cells are involved in this process. In the present paper we aimed to determine these interactions. Using thromboelastometry, we measured fibrin clot formation in whole blood and plasma under conditions of dilution *in vitro* or *in vivo*, and assessed the effects of reconstitution of blood cells (platelets, red blood cells) and plasma coagulation factors (fibrinogen, PCC). The results show that the clot-forming process is controlled by platelets and fibrinogen in additive and independent ways, implicating that the platelet count is an important functional variable, independently of fibrinogen, in whole-blood thromboelastometry under

condition of *in vivo* dilution in patients undergoing cardiothoracic surgery.

Materials and Methods

Materials

Bovine serum albumin (BSA) and apyrase were obtained from Sigma (St. Louis MO, USA). Human thrombin calibrator and thrombogram software were from Thrombinoscope (Maastricht, The Netherlands), thrombin substrate Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC) was from Bachem (Bubendorf, Switzerland). Thromboelastometry International (Munich, Germany) supplied the corresponding hardware, software and cuvettes.

Fibrinogen concentrate (Haemocomplettan P) and prothrombin complex concentrate (PCC, Beriplex) were from CSL-Behring (Marburg, Germany). PCC contains the vitamin K-dependent coagulation factors, prothrombin, factor VII, IX and X, and the anticoagulant factors, protein C and S, and antithrombin/heparin; 1 U/ml PCC refers to 100% prothrombin. Recombinant tissue factor (Innovin) was from Dade Behring (Deerfield IL, USA). Phospholipid vesicles containing phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (20:60:20) were purchased from Avanti (Alabaster AL, USA) and prepared by ultrasonication.[18] All other reagents came from sources described before.[5]

Blood donors

The studies were approved by the local medical ethics committee (MEC 07-2-114). Healthy donors and patients gave full informed consent for blood donation, according to the Helsinki declaration. Healthy subjects had not taken antithrombotic medication for at least two weeks. For *in vitro* dilution experiments, blood was obtained from 15 healthy subjects by venipuncture using a 1.2 mm needle, allowing the blood to drip freely into open tubes (first 2-3 ml were discarded). The collection tubes contained either 1/10 volume of trisodium citrate (0.129 M) for the preparation of platelet-free plasma; or 1/6 volume of acidic citrate dextrose (ACD: 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the isolation of washed platelets.

Blood samples were obtained from 48 patients before and after a cardiopulmonary bypass (CPB) procedure. This sample size is based on the results of an earlier study, where the effects of *in vivo* dilution on elastic fibrin clot formation in only plasma were

determined.[19] Patients were admitted in the hospital in the period of October to December 2010. Patients had stopped taking anticoagulant drugs at least 1 week before the procedure. Blood samples were collected into 0.129 M trisodium citrate (1:10) Vacuette tubes (Greiner, Alphen aan de Rijn, The Netherlands) at two time points: *(i)* after induction of anesthesia, but prior to the CPB procedure and heparin administration; *(ii)* after surgery and infusion of protamine to neutralize heparin, when the activated clotting time (ACT) was normalized. In samples from 4 patients coagulation times pointed to the presence of residual traces of heparin. In these cases, assays were repeated in the presence of 10 µg/ml polybrene, i.e. a concentration not influencing the assay parameters (data not shown). None of the patients developed surgical complications other than bleeding during the time span of blood sampling. Transfusion of blood products during surgery was guided on the basis of low blood cell counts, prolonged aPTT, and/or clinical observation of bleeding. Whole blood thromboelastometry was measured immediately after collection; plasma samples were stored for later measurements.

Preparation of plasma, washed platelets and red blood cells

Platelet-free plasma was obtained from citrate-anticoagulated blood, by centrifuging twice at 2630 g for 10 min.[20] Plasma samples were immediately snap-frozen into liquid nitrogen, and stored at -80°C until further use. Washed platelets were prepared from ACD-anticoagulated blood, and suspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA), as described.[19] Platelet count was determined with a thrombocounter (Coulter Electronics, Luton, UK). For the isolation of red blood cells, citrate-anticoagulated blood was centrifuged at 240 g for 15 min, after which the red cell layer was supplemented with Hepes buffer pH 7.45 in a 1:2 volume ratio. Red cells were then centrifuged at 2630 g for 10 min, suspended with Hepes buffer pH 7.45 (2:1), and washed again, which yielded a highly purified suspension of red cells (99.9%).

Dilution of whole blood or plasma and reconstitution experiments

Whole blood or plasma was diluted *in vitro* with saline medium, consisting of 137 mM NaCl, 12.9 mM trisodium citrate, 2 mM $CaCl_2$ and 2 mM $MgCl_2$, in order to keep equal concentrations of free Ca^{2+} and Mg^{2+} in all diluted samples. Coagulation factor levels in plasma were determined, as described.[5] Percentage values of plasma are given as final concentrations (relative to citrate-anticoagulated plasma taken as 100%). In all

reconstitution experiments, replacement of plasma by added supplements was taken into account when calculating the final extent of dilution. Red blood cells were added at 1:10 volume ratio. Where indicated, factor concentrates in saline were added to plasma samples from healthy subjects or patients, and incubated for 10 min before starting the experiment.

Rotational thromboelastometry

In samples from the same donors, elastic fibrin clot formation was measured in whole blood and plasma (citrate-anticoagulated) by thromboelastometry, using equipment and cuvettes from TEM International. Coagulation was triggered with 10 pM tissue factor and a surplus of $CaCl_2$.[19] Plasma samples were supplemented with phospholipid vesicles (4 μM, final concentration) or washed platelets from one healthy donor (100-250 × 10^9/l, final count). Runs were performed in duplicate or, when >5% variation was observed, in triplicate. Curves were analyzed for slope of elastic clot formation (α-angle), maximum strength of the clot (maximal clot firmness, MCF) and the time to the onset of clot formation (clotting time, CT).

Thrombin generation

Thrombin generation in plasma was measured, using the Calibrated Automated Thrombogram (CAT) method.[19] Plasma samples were supplemented with either phospholipid vesicles (4 μM) or washed platelets from a single healthy donor (100-250 × 10^9/l). Assays were run in 96-well U-bottom plates (Milford, MA, USA) in the presence of fluorogenic substrate Z-GGR-AMC (416.7 μM) and $CaCl_2$ (16.7 mM, final concentrations are given). Coagulation was triggered with 10 pM tissue factor. Measurements were performed in triplicate. First-derivate curves of thrombin generation were obtained by using Thrombogram software.[20] Curves were analyzed for maximal rate of thrombin generation (thrombin peak height), thrombin-generating capacity (endogenous thrombin potential, ETP) and the time until thrombin formation (lag time). Samples containing added PCC were analyzed for 10 min after triggering coagulation, to ensure adequate curve calibration.

Statistical analysis

Data are given as means ± SD. The Shapiro-Wilk test was used to test for normal distribution of the data. Statistical analysis was performed, as appropriate, using the Mann-Whitney *U* test. Patient data were not normally distributed and are given as

medians with interquartile ranges. The Wilcoxon matched pairs signed ranked test was used for comparing pre- and post-surgical values. Determinants of thromboelastometry curves were identified by multiple linear regression analysis. *P*-values <0.05 were considered statistically significant. The program GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for graphical purposes and relevant statistics; the SPSS 20 (IBM, Armonk, NY, USA) package was used for regression analysis and statistical testing.

Results

Determinants of thromboelastometry and thrombin generation curves for whole blood and plasma upon in vitro dilution

Blood samples from healthy subjects were diluted *in vitro*, reconstituted with various blood components, and analyzed for elastic fibrin clot formation by thromboelastometry. Coagulation was triggered via the extrinsic pathway with recombinant human tissue factor at an optimal concentration of 10 pM.[19] To keep cationic concentrations constant, citrate-anticoagulated blood samples were diluted with saline medium, containing equimolar concentrations of citrate, $CaCl_2$ and $MgCl_2$. The MCF, as a curve parameter determining the overall clotting capacity, was half-maximal at 30% blood, while the α-angle, detecting the rate of elastic clot formation, was half-maximal at 20% blood (Figure 1A). The clotting time (CT) was prolonged from <40% blood (Figure 1B). Strikingly, after dilution of blood to 40% (as in patients undergoing major surgery with bleeding), neither the MCF nor the α-angle changed by addition of PCC or red blood cells (Figure 1C). Addition of red blood cells to raise the hematocrit from 10 to 35% (at 40% plasma) did not influence thromboelastometry curve parameters (data not shown, but see below). In contrast, re-addition of either platelets (to 250 × 10^9/l) or fibrinogen concentrate (to 3.0 g/l) to the diluted blood markedly restored parameters of fibrin clot formation (Figure 1C). Only addition of platelets normalized the CT (Figure 1D).

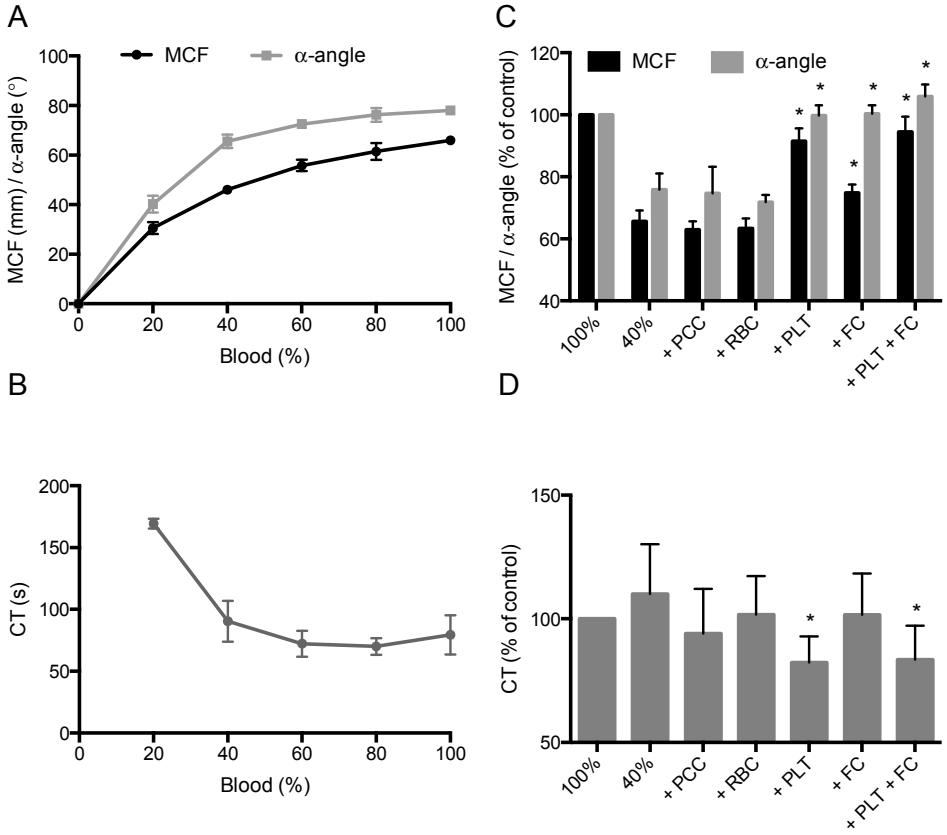

Figure 1: Determinants of thromboelastometry in diluted whole blood. Whole blood samples from healthy subjects were diluted with saline medium (see methods) to the indicated percentage. Levels of hemostatic factors in the undiluted blood (plasma) were: platelets 220-250 × 10^9/l; red blood cells 33-37% hematocrit; fibrinogen 3.0-3.5 g/l, prothrombin 100%. (A, B) Effect of blood dilution on maximal clot firmness (MCF), α-angle and clotting time (CT) of thromboelastometry curves. (C, D) Whole blood diluted to 40% was supplemented (final concentrations) with prothrombin complex concentrate (PCC 1 U/ml, equivalent to 100% prothrombin), red blood cells (RBC, 37% hematocrit), autologous platelets (PLT, 250 × 10^9/l) and/or fibrinogen concentrate (FC, 3.0 g/l.). Values of MCF, α-angle and CT are given in comparison to the control condition with undiluted blood (100%). Means ± SD (*n* = 5-8), **P* < 0.05 *vs*. no addition.

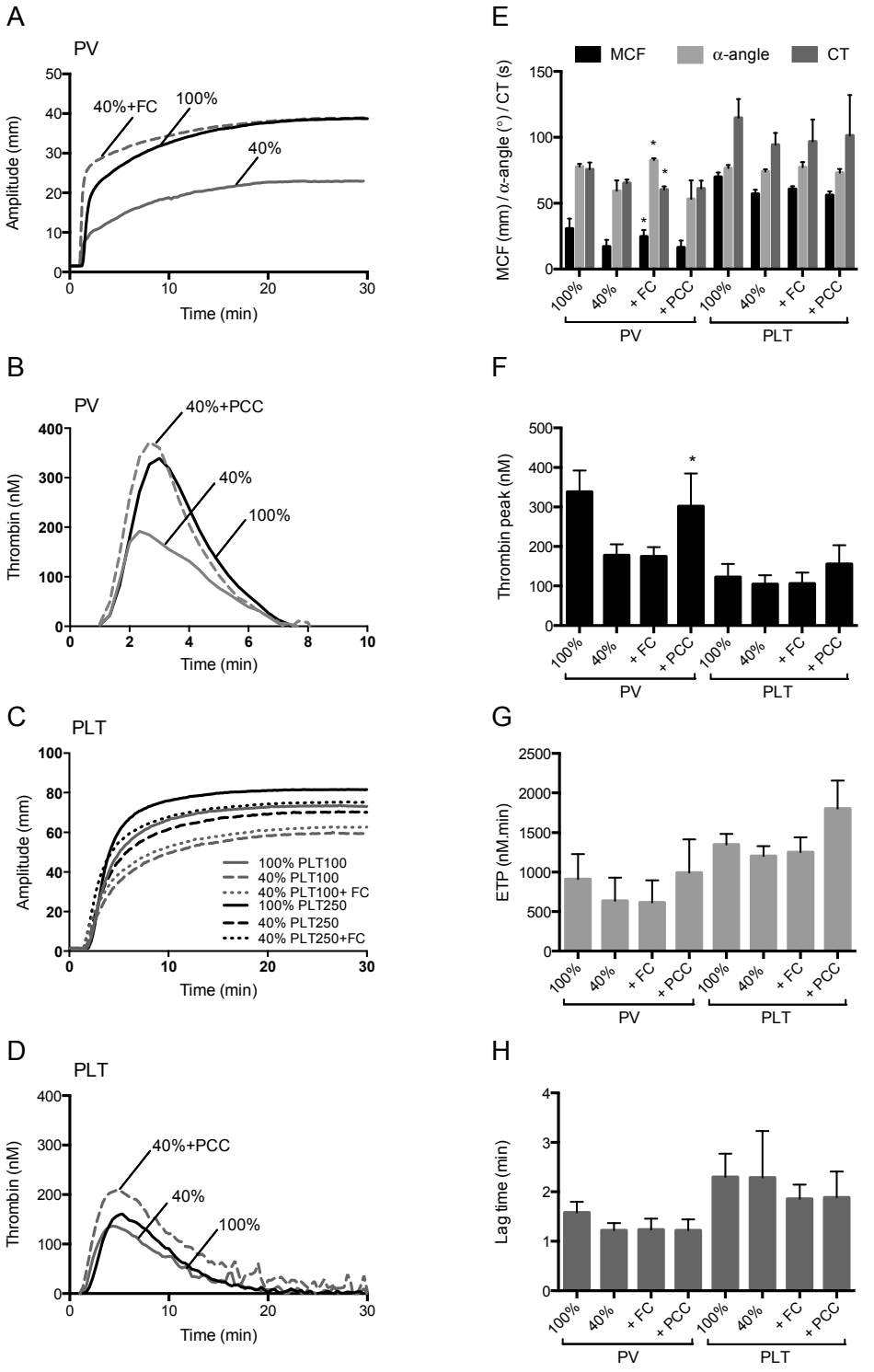
A
PV
40%+FC
100%
40%
Amplitude (mm)
Time (min)
B
PV
40%+PCC
40%
100%
Thrombin (nM)
Time (min)
C
PLT
100% PLT100
40% PLT100
40% PLT100+ FC
100% PLT250
40% PLT250
40% PLT250+FC
Amplitude (mm)
Time (min)
D
PLT
40%+PCC
40%
100%
Thrombin (nM)
Time (min)
E
MCF
α-angle
CT
MCF (mm) / α-angle (°) / CT (s)
100%
40%
+ FC
+ PCC
PV
PLT
F
Thrombin peak (nM)
G
ETP (nM.min)
H
Lag time (min)

Figure 2: Determinants of thromboelastometry and thrombin generation in diluted plasma. Normal plasma (fibrinogen 3.2 g/l, prothrombin 100%) was diluted with saline medium. Samples were replenished (final concentrations) with fibrinogen concentrate (FC, 3.0 g/l) or prothrombin complex concentrate (PCC, 100% prothrombin). Final plasma dilution of all samples was 40%. Coagulation was triggered with 10 pM tissue factor in the presence of either procoagulant phospholipid vesicles (PV, 4 µM) or platelets (PLT, 100 × 10^9/l). (A, C) Representative thromboelastometry curves with phospholipid vesicles or platelets. (B, D) Representative thrombin generation curves with phospholipid vesicles or platelets. (E-H) Effect of added fibrinogen or PCC on parameters of thromboelastometry (E) and thrombin generation curves (F-H). Means ± SD (*n* = 5-6), **P* < 0.05 *vs*. no addition.

Given the inability of red cells to affect thromboelastometry curves, further experiments were performed with isolated plasma, as this allowed simultaneous measurement of elastic clot formation and thrombin generation. Coagulation was triggered with 10 pM tissue factor, while phospholipid vesicles (4 µM) or autologous platelets (100-250 × 10^9/l) were added to provide a phospholipid surface for coagulation activity.[19] In the presence of phospholipid vesicles, thromboelastometry curves remained low in slope (α-angle) and amplitude (MCF). Plasma dilution to 40% resulted in further lowering of the curves (Figure 2A), and in diminished thrombin generation (Figure 2B). Supplementation of fibrinogen concentrate, but not of PCC, antagonized the dilution effect on thromboelastometry (Figure 2A, E). In contrast, only PCC markedly antagonized the dilution effect on thrombin generation (Figure 2B, F-H). This analysis underscored the fibrinogen level as a major variable (independently of platelets) in elastic clot formation.

In the presence of platelets (100 × 10^9/l), the rate and maximal amplitude of elastic clot formation were markedly higher and reached the maximal levels detected in whole blood (Figure 2C, E), thus pointing to an additive effect of platelets to the thromboelastometry curves. In this case, plasma dilution to 40% only moderately diminished the thromboelastometry curves. In measurements of thrombin generation with platelets, plasma dilution was of limited effect on thrombin peak height, total amount of thrombin (ETP) or lag time (Figure 2F-H).

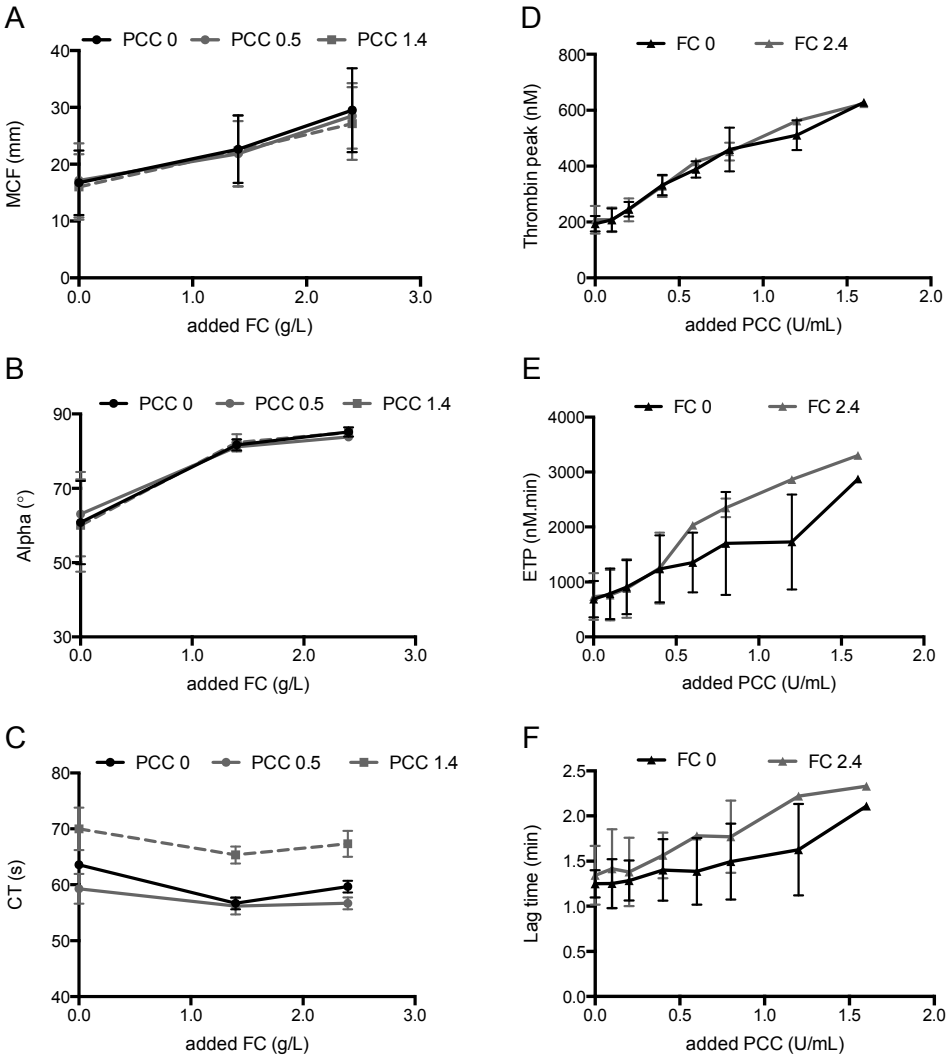

Figure 3: Dissimilar effects of fibrinogen and prothrombin complex concentrate on thromboelastometry and thrombin generation in diluted plasma. Normal plasma was diluted with saline medium and replenished with fibrinogen concentrate (FC, 1.4-2.4 g/l) and/or prothrombin complex concentrate (PCC, 0.1-1.6 U/ml). Final plasma dilution in all samples was 40%. Coagulation was triggered with 10 pM tissue factor in the presence of phospholipid vesicles. Effects of addition of different amounts of FC with or without PCC (0.5/1.4 U/ml) on MCF (A), α-angle (B) and CT (C) of the thromboelastometry curves. Effects of addition of different amounts of PCC with or without FC (2.4 g/l) on peak height (D), ETP (E) and lag time (F) of the thrombin generation curves. Means ± SD (*n* = 3-6).

Dose-response studies were performed to better define the effects of fibrinogen and PCC in diluted plasma. For thromboelastometry curves, added fibrinogen up to 3-4 g/l (final concentration) caused a dose-dependent increase in MCF and α-angle, regardless of whether PCC was added or not (Figure 3A, B). Added fibrinogen did not affect the CT, while only the highest concentration of PCC caused a slight prolongation in CT (Figure 3C). Addition of PCC resulted in a dose-dependent increase in thrombin generation, reaching 300% of the normal thrombin peak height and ETP level. At none of the doses, fibrinogen influenced parameters of thrombin generation (Figure 3D, E). However, PCC at high doses prolonged the lag time to thrombin formation (Figure 3F), which can be explained by the presence of anticoagulant proteins in this concentrate. Together, these data indicate that, in diluted whole blood and plasma, fibrin clot formation relies on the amounts of platelets and fibrinogen, whereas the thrombin generation process is regulated by the levels of vitamin K-dependent coagulation factors, as present in PCC.

In thromboelastometry, effects of varying the levels of both platelets and fibrinogen were compared. These blood components were added in various combinations to diluted plasma, while keeping the final extent of dilution constant. The three-dimensional plot of Figure 4A indicates that, up to 250 × 10^9 platelets/l and 2.7 g fibrinogen/l, the MCF increased with higher levels of both platelets and fibrinogen (Figure 4A). Even at the highest fibrinogen concentrations, the MCF raised with the platelet count. This is also apparent from a plot of the α-angle, although this parameter reached a maximal value at lower fibrinogen and platelet levels (Figure 4B). Regression analysis revealed that the platelet count contributed more strongly to the MCF than the fibrinogen level; with a standardized regression coefficient β of 0.760 and 0.498, respectively (Suppl. Table 1). Furthermore, the relative contribution of fibrinogen to MCF appeared to be independent of the platelet count, *i.e.* standardized regression coefficients for fibrinogen were >0.89. This was also true for the α-angle.

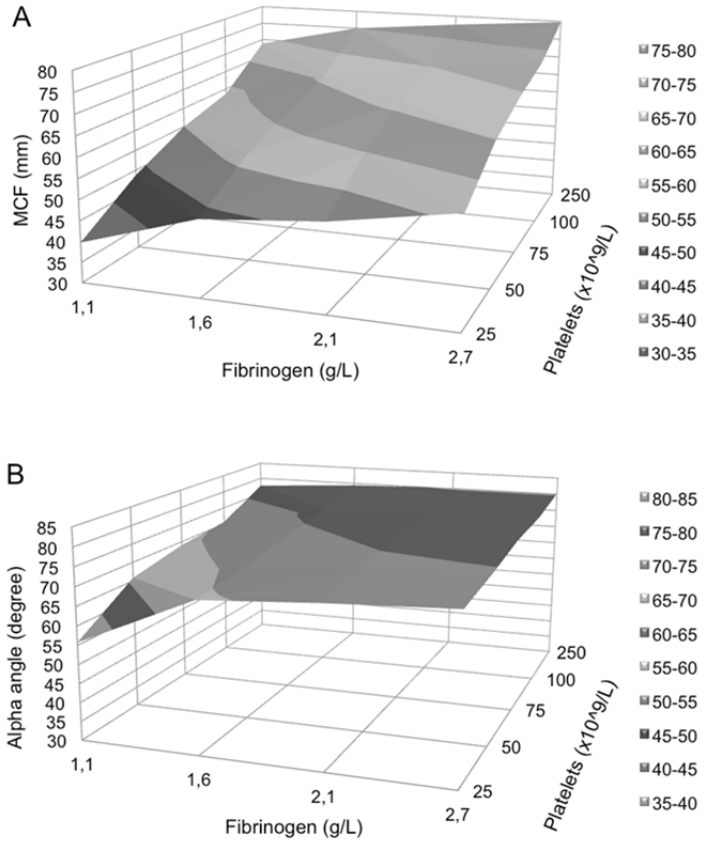

Figure 4: Combined contribution of fibrinogen and platelets to thromboelastometry. Plasma was diluted as in Figure 3; fibrinogen levels were adjusted to 1.1-2.7 g/l, while platelets were added at 25-250 × 10^9/l. Thromboelastometry curves were analyzed, as described. Graphs show effect of fibrinogen and platelet variation on MCF (A) and α-angle (B). See also Suppl. Table 1.

Predictive variables of whole blood thromboelastometry and thrombin generation in patients undergoing hemodilution during surgery

To assess the clinical relevance of these different roles of platelets and fibrinogen in thromboelastometry, blood and plasma samples were studied from patients undergoing cardiothoracic surgery and *in vivo* dilution by massive fluid infusion. Blood samples were analyzed from 48 patients, with mean age of 67 (range 51-75) years, of whom 33 underwent coronary artery bypass grafting and 10 replacement of the aortic or mitral valve (Suppl. Table 2). Total fluid volume transfused during the surgery procedure was 4.6 ± 1.4 l (mean ± SD). A minority (12 patients) needed transfusion with 2.2 ± 1.5 units packed red cells. Blood samples were obtained from the patients

before and after the surgical procedure. All patients received heparin (344 ± 77 mg) after collection of the first blood sample, and the heparin was antagonized with protamine (269 ± 51 mg) before the second blood collection. Plasma samples were checked for absence of residual heparin activity.

Table 1: Hematological parameters of patients before and after cardiothoracic surgery. Blood was obtained from 48 patients before and after a CPB procedure. Inactivation of heparin was checked in all post-CPB samples. Mean changes (**Δ**) due to the surgery procedure are indicated in percentages. Medians with interquartile ranges (*n* = 48). ***P* < 0.001 *vs.* pre-surgery.

Parameter	Pre-surgery	Post-surgery	Δ
Platelets (× 10^9/l)	199 (159-227)	110 (83.3-140)**	*-44.7%*
Hematocrit (ml/ml)	0.32 (0.30-0.36)	0.24 (0.23-0.26)**	*-25.0%*
Hemoglobin (mM)	6.70 (6.10-7.50)	5.00 (4.68-5.53)**	*-25.4%*
aPTT (s)	30.0 (28.0-30.0)	35.0 (32.0-38.0)**	*+16.7%*
Prothrombin (%)	103 (87.0-118)	55.5 (43.5-64.0)**	*-46.1%*
Antithrombin (%)	98.0 (90.5-109)	57.5 (52.0-65.8)**	*-41.3%*
Factor X (%)	86.0 (74.5-109)	47.5 (42.3-58.0)**	*-44.8%*
Fibrinogen (g/l)	3.50 (2.90-4.30)	1.90 (1.50-2.30)**	*-45.7%*

In the group of patients, the platelet count dropped with 45%, the hematocrit level reduced with 25%, and the aPTT significantly prolonged from 30.0 (median) to 35.0 sec (Table 1). Plasma levels of prothrombin, antithrombin and factor X reduced with 41-46%, while fibrinogen decreased from 3.50 (median) to 1.90 g/l. In remaining plasma samples from a subgroup of 7 patients, levels of other coagulation factors were determined; factors VII, VIII and IX were reduced by 46%, 32% and 38%, respectively. Overall, this pointed to a dilution of ~40% of most blood components due to fluid infusion.

Whole blood samples pre- and post-surgery from all patients were analyzed by thromboelastometry upon triggering with tissue factor (Table 2). As expected, curves of clot formation narrowed significantly, in that the MCF decreased from median 66.0 (62.0-70.0) to 55.0 (48.0-60.0) mm, while the α-angle decreased from 76.0 (74.0-79.0) to 70.5 (63.4-71.80) degrees. Both the CT and clot formation time (CFT) significantly prolonged. During the 30 minutes of measurement, no signs of fibrin degradation were observed (not shown).

Table 2. Parameters of thromboelastometry in whole blood from patients before and after surgery. Thromboelastometry was determined in whole blood from 48 patients pre- and post-surgery, as described in the methods section. Data are medians with interquartile ranges. *$P < 0.05$, **$P < 0.001$ *vs.* corresponding pre-surgery.

Parameter	Pre-surgery	Post-surgery	Δ
MCF (mm)	66.0 (62.0-70.0)	55.0 (48.0-60.0)**	-16.7%
Alpha angle (°)	76.0 (74.0-79.0)	70.5 (63.4-72.8)**	-7.2%
CT (s)	112 (98.0-143)	135 (117-156)*	+20.5%
CFT (s)	70.0 (57.0-85.0)	104 (88.0-138)**	+48.6%

Multiple regression analysis demonstrated that in pre-surgery as well as in post-surgery samples, the platelet count and fibrinogen level were significant predictors of whole blood thromboelastometry. Standardized regression coefficients indicated that platelet count and fibrinogen level contributed similarly to the variation in MFC (Table 3). In contrast, variation in red blood cell count (hematocrit) or prothrombin level did not significantly add to this variation in either pre-surgery or post-surgery samples ($P > 0.1$).

Thromboelastometry and thrombin generation were also determined in plasma samples prepared from pre- and post-surgery blood (Table 4). Post-surgery thromboelastometry curves, obtained with plasma containing phospholipid vesicles, showed a consistent reduction in MCF (-46%) and α-angle (-11%) and a prolongation of the CT (+30%). These parameters also changed with platelets present. Analysis of thrombin generation measurements indicated a marked dilution effect post-surgery on thrombin peak height (-28%) and ETP (-7%), particularly when assessed with phospholipids. Exclusion of the data from patients, who had received red blood cells (n=12), resulted in similar changes of thromboelastometry and thrombin generation data (data not shown).

Table 3. Predicting variables of thromboelastometry in whole blood from patients before and after surgery. Fibrin clot formation was measured in whole blood from 48 patients pre- and post-surgery. Predicting variables of thromboelastometry MCF were obtained by linear, multiple regression analysis. Abbreviations: b indicates unstandardized regression coefficient; SE b, standard error of b; β, standardized regression coefficient.

	b	SE b	β	P-value	Partial correlation
Pre-surgery					
Step 1					
Constant	45.59	3.985		<0.001	
Fibrinogen	2.655	0.986	0.382	0.011	0.424
Platelets	0.054	0.018	0.422	0.005	0.460
Step 2					
Constant	44.07	9.627		<0.001	
Fibrinogen	3.031	1.079	0.436	0.009	0.450
Platelets	0.062	0.021	0.485	0.006	0.471
Hematocrit	7.989	23.92	0.047	0.741	0.060
Prothrombin	-0.042	0.044	-0.152	0.348	-0.169
Post-surgery					
Step 1					
Constant	33.805	2.505		<0.001	
Fibrinogen	5.783	1.296	0.507	<0.001	0.608
Platelets	0.080	0.021	0.439	<0.001	0.552
Step 2					
Constant	27.95	7.122		<0.001	
Fibrinogen	5.491	1.502	0.481	0.001	0.543
Platelets	0.080	0.025	0.440	0.003	0.498
Hematocrit	21.06	25.14	0.084	0.408	0.147
Prothrombin	0.024	0.073	0.048	0.749	0.057

To determine the potential for normalization, post-surgery plasma samples were supplemented with fibrinogen concentrate and PCC. These concentrates were added at amounts corresponding to the expected effect of transfusion of 2 units fresh frozen plasma[5], *i.e.*, 0.4 g/l fibrinogen plus 0.11 U/ml PCC. This addition significantly increased MCF in the presence of phospholipids from 16.5 (12.5-20.0) to 21.0 (17.0-25.0) mm, but left the parameter unchanged in the presence of platelets (Table 4). The same addition caused a 25-33% increase in thrombin generation (thrombin peak height and ETP), both with phospholipids and platelets.

Table 4: Comparison of thromboelastometry and thrombin generation tests in patient plasmas before and after surgery. Plasma samples from patients, before and after surgery, were supplemented with either 4 μM phospholipid vesicles or 100 × 10^9 platelets/l, as indicated. Coagulation was triggered with 10 pM tissue factor. Parallel plasma samples post-surgery were supplemented with fibrinogen concentrate (FC, 0.4 g/l, f.c.) and prothrombin complex concentrate (PCC, 0.11 U/ml, f.c.). Medians with interquartile ranges (n = 48). *P < 0.05, **P < 0.001 *vs.* pre-surgery samples, or *vs.* no addition.

Parameter	Pre-surgery	Post-surgery	Δ	Post + FC/PCC
Thromboelastometry in plasma				
Phospholipid vesicles				
MCF (mm)	30.5 (26.0-37.0)	16.5 (12.5-20.0)**	*-46%*	21.0 (17.0-25.0)**
Alpha angle (°)	79.0 (77.0-81.0)	70.0 (63.0-74.0)**	-11%	74.0 (71.0-78.0)**
CT (s)	89.0 (83.0-103)	116 (100-132)**	+30%	90.5 (77.0-105)**
Platelets				
MCF (mm)	73.0 (71.0-76.0)	65.0 (62.0-69.0)**	-11%	63.5 (59.0-66.0)
Alpha angle (°)	79.0 (77.0-80.0)	77.0 (75.0-78.0)**	-2.5%	76.5 (74.0-79.0)
CT (s)	104 (93.0-135)	111 (97.0-127)	+6.7%	95.5 (85.0-112)**
Thrombin generation in plasma				
Phospholipid vesicles				
Peak height (nM)	297.0 (249-325)	213.6 (179-232)**	-28%	269.5 (230-295)**
ETP (nM × min)	1159 (997-1313)	1083 (922-1252)**	-6.6%	1415 (1291-1563)**
Platelets				
Peak height (nM)	144.5 (114-168)	133.6 (113-151)*	-7.5%	167.1 (147-191)**
ETP (nM × min)	1282 (1094-1412)	1159 (1041-1358)*	-9.6%	1536 (1423-1805)**

The MCF of whole blood thromboelastometry with pre- and post-surgery samples was dependent on the fibrinogen level (partial correlation coefficients 0.917 and 0.890, respectively, P < 0.001). Notably, the MCF from curves obtained with whole blood samples correlated significantly with the MCF measured with plasma plus phospholipids (R = 0.83, P < 0.001) or with plasma plus platelets (R = 0.75, P < 0.001) (Figure 5). On the other hand, the various parameters of thromboelastometry curves (whole blood or plasma) did not correlate with those of thrombin generation (P > 0.2). Together, this indicated that the predictive variables of thromboelastometry curves of patient whole blood and plasma were similar, but differed from those predicting thrombin generation curves.

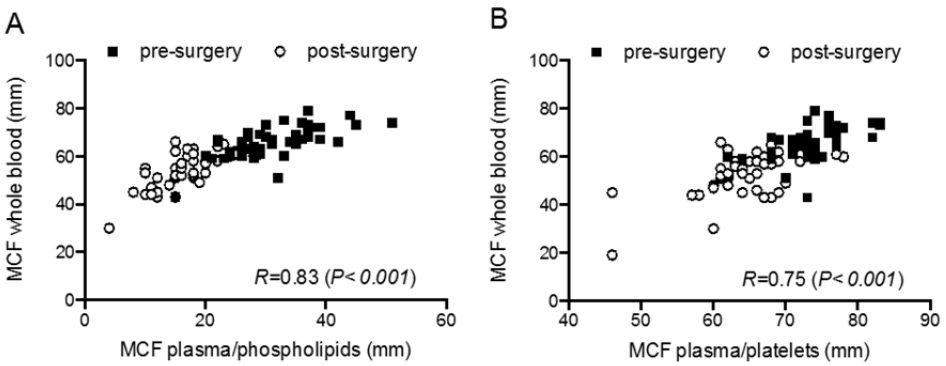

Figure 5: High correlation of thromboelastometry MCF values in whole blood and plasma samples. Thromboelastometry was measured in whole blood and plasma with phospholipids or platelets (100×10^9/l) from 48 patients. MCF was derived from the thromboelastometry curves. (A) MCF in whole blood *vs.* plasma/phospholipids, (B) MCF in whole blood *vs.* plasma/platelets. Correlation coefficients and P-values are given of combined pre- and post-surgery data.

Discussion

In this paper, we studied principal sources of variation of the processes of elastic fibrin clot formation (thromboelastometry) and thrombin generation under conditions of *in vitro* or *in vivo* dilution. Coagulation in all cases was fully activated with tissue factor.[21] It appeared that, in whole blood or plasma diluted *in vitro* to 40%, both the platelet count and fibrinogen concentration determine the kinetics and extent of fibrin clot formation in additive ways, with no more than limited contributions of red blood cells and vitamin K-dependent coagulation factors. In other words, certain (threshold) values of thromboelastometry parameters could be obtained by supplementation of platelets and fibrinogen at different relative amounts, but not by red blood cells. On the other hand, the best way to restore impaired thrombin generation in diluted plasma was by increasing the levels of vitamin K-dependent coagulation factors.

In the present paper we find that, under conditions of hemodilution, thromboelastometry parameters in whole blood are not improved by raising the hematocrit. A limited contribution of red blood cells is also apparent from the observation that curve parameters were similar in whole blood and in platelet-containing plasma (without red blood cells). Other authors, using undiluted blood have reported a negative contribution of the hematocrit on thromboelastometry parameters.[22-24] Comparing with the present results, this suggests that under high

viscous conditions, as in undiluted blood, red blood cells interfere with the formation of an elastic platelet-fibrin clot, while this interference becomes smaller under less viscous conditions.

The recognition that platelets and fibrinogen contribute to thromboelastometry parameters in additive ways sheds new light on our earlier conclusion[19], that platelets can partly compensate for the dilutional effect on elastic clot formation. The apparently independent contribution of platelets and fibrinogen most likely reflects differences in function in this process, *i.e.* providing elasticity to the fibrin clot by contraction (platelets) and by determining the mass of a fibrin clot (fibrinogen).[25] This suggestion is supported by the finding that also in blood samples from patients subjected to *in vivo* dilution, the platelet count and fibrinogen level are independent variables of the clot-forming process.

Marked differences were found, when comparing the effects of factor concentrates on thromboelastometry and thrombin generation under conditions of dilution *in vitro* or *in vivo*. Supplementation of fibrinogen concentrate, but not of PCC, in a dose-dependent way restored elastic fibrin clot formation, as detected by an increased MCF and α-angle, without affecting thrombin generation. Interestingly, the CT was hardly changed by PCC addition to whole blood or plasma, which agrees with published findings that the CT prolongs when coagulation factors fall below 35% of normal.[26] Conversely, supplementation of PCC, but not of fibrinogen concentrate, restored thrombin generation. These data indicate that, although thromboelastometry is often viewed as an integrated global measure of coagulation, its sensitivity to reduced levels of vitamin K-dependent coagulation factor is limited.

Human and large animal studies indicate that both types of factor concentrates can improve hemostasis and reduce blood loss after major trauma.[4, 15-17] In porcine models, infusion of a factor concentrate, not containing anticoagulant proteins, in contrast to fibrinogen concentrate, promoted disseminated intravascular coagulation and thus caused a prothrombotic condition.[27, 28] In vitro analysis indeed indicates that the absence of anticoagulant factors in such concentrates promotes thrombin generation.[29] In the present study, we find a prolonged CT (thromboelastometry) and lag time (thrombin generation) in the presence of a high concentration of PCC, which hence reflects the presence of anticoagulant factors that delay the formation of thrombin.

In patient plasmas after surgery, parameters of thrombin generation were less strongly reduced than those of thromboelastometry. Impairment in thrombin generation is

demonstrated by the reduction in thrombin peak level as well as by the prolongation of the CT. Comparison of the curve parameters learned that the thrombin peak height was a better responsive indicator for dilution than the ETP. The latter agrees with other papers reporting on effects of *in vitro* dilution in thrombin generation.[21, 30]

Taken together, we conclude that the platelet count and fibrinogen level, in additive ways, but not the amount of red blood cells, determine the extent of elastic fibrin clot formation in diluted whole blood or plasma. Thrombin generation in diluted plasma, on the contrary, is dependent on vitamin K-dependent coagulation factors. By implication, clinical decisions for transfusion with fibrinogen or plasma that are based on thromboelastometry should take into account the platelet concentration.

Acknowledgments

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Supplementary materials

Supplementary Table 1: Relative contribution of platelets and fibrinogen to thromboelastometry curves. PRP was reconstituted to the indicated platelet number (x 10^9/l) and the relative contribution of fibrinogen to the MCF and alpha angle was analyzed by linear regression analysis. Abbreviations: *b,* unstandardized regression coefficient; *SE b,* standard error of *b*; β standardized regression coefficient (n = 5).

		b	***SE b***	**β**	***P-value***
MCF					
Plt 25	Constant	30.82	1.850		<0.001
	Fibrinogen	9.700	0.943	0.924	<0.001
Plt 50	Constant	39.54	2.087		<0.001
	Fibrinogen	9.663	1.064	0.906	<0.001
Plt 75	Constant	46.16	1.451		<0.001
	Fibrinogen	9.064	0.739	0.945	<0.001
Plt 100	Constant	50.28	1.608		<0.001
	Fibrinogen	8.839	0.820	0.931	<0.001
Plt 250	Constant	62.84	1.562		<0.001
	Fibrinogen	6.667	0.796	0.892	<0.001
Alpha					
Plt 25	Constant	48.80	2.961		<0.001
	Fibrinogen	9.925	1.509	0.840	<0.001
Plt 50	Constant	61.20	1.300		<0.001
	Fibrinogen	5.431	0.662	0.888	<0.001
Plt 75	Constant	64.74	1.096		<0.001
	Fibrinogen	4.981	0.559	0.903	<0.001
Plt 100	Constant	67.64	1.288		<0.001
	Fibrinogen	4.232	0.656	0.835	<0.001
Plt 250	Constant	76.34	1.040		<0.001
	Fibrinogen	1.610	0.530	0.582	0.007

Supplementary Table 2: Patient and surgery characteristics. Means ± SD (n = 48).

Characteristic	
Male/female (n)	32/16
Mean age in years (range)	67 (51-75)
Medication prior to surgery:	
Aspirin (n)	28
Coumarin (n)	2
Fraxiparine (n)	5
Type of surgery:	
Coronary artery bypass graft (n)	33
Valve replacement (n)	10
Other (n)	5
Heparin during surgery + protamine (n)	48
Perfusion time (min)	91 ± 31
Type of transfusion (n)	
Red blood cells (n)	12
Fresh frozen plasma (n)	0
Fluids (n)	48
Transfused fluids:	
Priming fluid (l)	1.4 ± 0.4
Cardioplegia (l) (n=11)	1.9 ± 0.8
Colloids/crystalloids (l)	1.8 ± 0.8
Total (l)	4.6± 1.4

Chapter 8

Perioperative dilutional coagulopathy treated with fresh frozen plasma and fibrinogen concentrate: a prospective randomized intervention trial

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Abstract

Background: Treatment of dilutional coagulopathy by transfusing fresh frozen plasma (FFP) remains sub-optimal. We hypothesized that partial replacement of transfused FFP by fibrinogen concentrate results in improved coagulant activity and haemostasis. This was tested in a controlled clinical intervention trial with patients experiencing massive bleeding during major surgery.

Methods: Patients undergoing major elective surgery were treated according to current protocols. When transfusion with FFP was required, patients were randomized as follows: group A received 4 units FFP; group B received 2 units FFP plus 2 g fibrinogen concentrate. Blood samples were taken before and after the intervention. Analysts were blinded to the treatment type.

Results: Group A (B) consisted of 21 (22) patients, in 16 (17) of whom bleeding stopped after intervention. Plasma fibrinogen increased significantly more in group B (0.57 g/L) than in group A (0.05 g/L). However, levels of prothrombin, factors VIII, IX and X increased more in group A than in group B. Rotational thromboelastometry (ROTEM) of whole blood and plasma revealed improved fibrin clot formation in group B but not in group A. Thrombin generation (Calibrated Automated Thrombogram, CAT) in plasma increased more in group A. Principal parameters determining whole-blood thromboelastometry were the fibrinogen level and platelet count. *In vitro* addition of fibrinogen and prothrombin complex concentrate to pre-intervention samples restored both ROTEM and CAT parameters.

Conclusions: Partial replacement of transfused FFP by fibrinogen increases fibrin clot formation at the expense of less improved thrombin generation. Coagulation factors other than fibrinogen alone are required for full restoration of haemostasis.

Introduction

In major elective surgery and trauma, successful treatment of massive haemorrhage remains a challenge, as bleeding is still the main cause of morbidity and mortality in these situations.[1, 2] Patients undergoing major surgery develop haemodilution, as a consequence of abundant infusion of fluids for volume resuscitation and transfusion of blood components. The result often is diminished coagulation, which further aggravates the bleeding risk. Transfusion of blood components may also lead to other complications, such as acute respiratory distress syndrome, transfusion-related lung injury, transfusion-associated circulatory overload and infections.[3, 4] Recent association studies provide indications that the use of multiple blood components reduces long-term survival after cardiac surgery.[5, 6] Accordingly, alternatives for blood component transfusion, for example the use of factor concentrates, are extensively being studied.

Current guidelines for major surgery recommend transfusion therapy with crystalloids, colloids, packed red cells and fresh frozen plasma (FFP) to prevent shock and restore the coagulation processes.[7, 8] Colloids yet affect haemostasis by disturbing fibrinogen polymerization,[9, 10] while also the clinical efficacy of FFP in preventing peri-operative bleeding is debated.[11, 12] An interesting alternative to FFP is transfusion with fibrinogen concentrate, especially since fibrinogen is the first coagulation factor falling below a critically low level upon dilution.[13] Fibrinogen concentrates have appeared to be haemostatically active in both retrospective clinical studies and animal models.[14-17] In this regard, whole-blood rotational thromboelastometry is used as a valuable point-of-care method, monitoring improved fibrin clot formation upon elevation of plasma fibrinogen levels.[13, 18] However, the precise indications for fibrinogen transfusion and the limitations of this intervention are still debated.[19, 20] Recently, we reported that the processes of thrombin generation and fibrin clot formation are independently reduced during dilutional coagulopathy.[9, 21] The implication is that full replacement of transfused FFP by fibrinogen concentrate may not result in haemostasis, as levels of other coagulation factors than fibrinogen remain too low for a critical rate of thrombin generation.

In this paper we present a first prospective randomized intervention study aimed to compare the effect of partial replacement of FFP by fibrinogen concentrate on fibrin clot formation, as measured by whole blood thromboelastometry, and thrombin generation in patients with massive bleeding undergoing major elective surgery.

Materials and Methods

Study setup

The study was designed as a single-centre, prospective, randomized, blinded intervention trial, and was approved by the medical ethical board of the Maastricht University Medical Centre+ (trial registration NL23565.068.08). Patients selected as eligible prior to surgery, gave written informed consent to participate. Selection criteria were the type of operation (cardiovascular surgery, major abdominal surgery or orthopedic surgery) and the expected duration of operation (>120 min). Exclusion criteria were age (<18 years), active HIV infection, known coagulation abnormalities, deep hypothermia with circulatory arrest, or preoperative need of transfusion.

Haemostatic therapy to stop bleeding was started, based on clinical decision. Massive bleeding was defined as prolonged blood loss of >150 mL/h or >1.5 mL/kg/20 min, or acute blood loss of >700 mL at once.[7, 19, 20] Patients with massive bleeding during or after surgery were randomized to group A or B in the transfusion laboratory, using a closed envelope method. Products were prepared and sent to the operation theatre, where the attending anaesthesiologist managed the transfusion. Patients of group A were transfused with 4 units FFP, those of group B with 2 units FFP plus 2 g fibrinogen (Haemocomplettan P 20 g/L, CSL Behring, Marburg, Germany).

Blood samples were taken directly before (baseline) and immediately after completion of the transfusion products. Heparin (150-300 mg/kg) was given to 12 out of 13 (group A) and 13 out of 14 patients (group B), who required cardiovascular surgery with extracorporeal circulation. Before taking the second blood sample, the heparin was antagonized with protamine in a ratio of 1:1, verified by the activated clotting time (ACT). Extra protamine was given, if the baseline ACT was not reached. In few (5) plasma samples with traces of active heparin, that is, in patients with prior heparin medication, functional measurements were performed in the presence of 10 U/mL polybrene (Janssen, Beerse, Belgium).

Criteria for stopped bleeding after transfusion were as follows: no visual blood loss or clotting, no monitored circulatory instability, and/or no major decrease in haematocrit. When haemostasis remained insufficient, therapy was continued according to standard treatment protocols. Demographic data, type of surgery, duration of the operation, peri-operative fluid management, temperature and peri-operative morbidity of the patients were all documented.

Plasma preparation

Blood was drawn into sodium citrate (1/10 volume, 10.9 mM f.c.). Haematological parameters were determined as described.[21] Platelet-free-plasma (PFP) was prepared by centrifuging blood samples twice at 2630 *g* for 10 min at room temperature. Collected plasma was aliquoted and snap-frozen at -80°C until use. To prepare washed platelets, blood from a healthy donor was collected into acetate-citrate dextrose and subjected to a centrifugation procedure.[21]

Plasma supplementation with factor concentrates

To study effects on thromboelastometry and thrombin generation, pre-intervention plasma samples were supplemented with fibrinogen concentrate and prothrombin complex concentrate. The concentrates were added at amounts corresponding to an expected increase in fibrinogen and prothrombin level reached by transfusion of 4 units FFP. Using a published algorithm,[9] this required the addition of 0.8 g/L fibrinogen concentrate (Haemocomplettan P) and/or 0.22 U/mL prothrombin complex concentrate (Beriplex, CSL-Behring). After reconstitution, the plasmas were incubated at 37°C for 10 min.

Coagulation times and factor measurements

Prothrombin time (PT) was measured by triggering plasma with tissue factor (Innovin, Dade Behring, Marburg, Germany). The activated partial thromboplastin time (aPTT) was measured with the actin-FSL kit (Dade Behring). Fibrinogen was determined with a functional assay.[22] Plasma prothrombin and factors VIII, IX and X were determined with one-stage clotting assays using the Behring coagulation system (Dade Behring). Antithrombin was measured with a chromogenic assay in the same system. Plasma heparin was assessed as anti-factor Xa activity using the Coamatic heparin test (Chromogenics, Mölndal, Sweden).

Rotational thromboelastometry

Rotational thromboelastometry was performed with whole blood and plasma using a ROTEM thromboelastometer (TEM International, Munich, Germany). Freshly obtained, citrate-anticoagulated whole blood (300 µL) was activated with an optimal concentration of 10 pM tissue factor and $CaCl_2$. Because of late availability of the equipment, ROTEM analysis was not performed with whole blood samples from the first patients entering the study. For thromboelastometry experiments with plasma,

snap-frozen PFP was thawed, and 300 µL aliquots were supplemented with donor platelets (100×10^9/L) or 4 µM phospholipids (Phosphatidyl serine, phosphatidyl choline and phosphatidyl ethanolamine, 1:3:1; mol:mol, Avanti, Alabaster AL, USA).
Activation was with 10 pM tissue factor and $CaCl_2$.[17] Evaluation parameters of the curves were the clotting time (CT), clot formation time (CFT), maximal clot formation (MCF) and maximal rate of clot formation (α-angle). The two latter inform on the maximal elastic clot strength and the rate of fibrin strand formation, respectively.[26] Normal value of the MCF of PFP with phospholipids was 42 ± 5.7 mm (TEG, mean ± SD, *n*=40); the preset cut-off level of normal fibrin clot formation was 13.5 mm.[9]

Thrombin generation measurement.
Thrombin generation was measured using the calibrated automated thrombogram method (CAT; Thrombinoscope, Maastricht, the Netherlands), with slight modifications.[17, 21] Citrate-anticoagulated PFP was supplemented with donor platelets (100×10^9/L) or phospholipids (4 µM), and activated with 10 pM tissue factor plus $CaCl_2$. Thrombin activity at 37°C was determined from the cleavage of thrombin substrate, Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC, Bachem, Bubendorf, Switzerland). Samples were run in triplicate. Evaluated curve parameters were the thrombin peak height and endogenous thrombin potential (ETP, area-under-the-curve).[27] The intra-assay variation coefficient was 6%. Normal value of the thrombin peak height in PFP with phospholipids was 314 ± 24 nM (mean ± SD, *n*=40); the cut-off level for normal thrombin generation was 194 nM.[9]

Statistical analysis.
Data are expressed as means ± SD. Data from study groups were compared by ANOVA. Treatment effects on assay parameters (normally distributed) were assessed with a paired t-test (2-tailed) and Pearson correlation analysis. The statistical package for the social sciences was used (SPSS, version 15.0; SPSS Inc., Chicago, IL, USA).

Results

During the study period of 17 months, 307 eligible patients gave informed consent prior to surgery. During surgery, 255 did not meet the predefined criteria for massive bleeding. Of the remaining 52 patients with bleeding, 9 patients required transfusion

of only 2 units FFP and hence were not included. The remaining 43 patients with massive bleeding entered the study protocol, and received either 4 units FFP (group A) or 2 units FFP plus 2 g fibrinogen (group B).

Group A consisted of 21 patients (15 men and 6 women) with mean age of 65.2 years; group B contained 22 patients (16 men and 6 women) with mean age of 65.6 years (Table 1). In either group, the majority of patients underwent cardiovascular surgery (13 and 14, respectively), while the other patients had abdominal (7 and 6) or spinal column surgery (1 and 2). Patients of groups A and B did not differ with respect to the length of operation. The volume of infused colloids (HES 6% 130/04) was similar for both groups. Also the total fluid volume transfused prior to intervention was similar: 8.51 ± 2.64 L for group A and 8.72 ± 4.13 L for group B (mean ± SD). After intervention, haemostasis was sufficient in 16 patients of group A and 17 of group B (p=0.93). In the remaining patients with continued bleeding, five per group, prohaemostatic treatment was continued according to the standard hospital protocol. One patient with bleeding in either group showed pleural effusion, 3 patients of group A had wound infections (one associated with deep vein thrombosis), while in group B, one patient had wound infections, two had septic complications (one pneumonia treatment), and one abdominal ischemia needing surgical revision.

Table 1: Patient and surgery characteristics of groups A and B.

	Group A	**Group B**
Male/female (*n*)	15/6	16/6
Age (years)	65.2 (9.8)	65.6 (10.7)
Surgery type: (*n*)		
Cardiovascular	13*	14*
Abdominal	7	6
Spinal column	1	2
Surgery duration (min)	374 (126)	355 (96)
Temperature pre-intervention (°C)	35.9 (1.2)	35.8 (1.1)
Transfusion before intervention:		
Packed red cells (units)	6.33 (2.80)	5.86 (3.54)
Colloid HES 130/0.4 (L)	1.89 (0.89)	2.00 (0.87)
Total fluid volume (L)	8.51 (2.64)	8.72 (4.13)

Total fluids transfused prior to intervention comprise colloids, crystalloids and priming and cell saver fluids. Data are means (SD). *12/13 patients (group A) and 14/14 patients (group B) received 3 g tranexamic acid.

Regarding haematological parameters, haemoglobin and platelet count were similar in both groups, without significant changes after intervention (Table 2). Plasmas from both groups showed a prolongation of the aPTT, but not the PT. After intervention, the aPTT significantly shortened more in group A than in group B (p=0.043), while fibrinogen levels increased only in group B. Plasma levels of prothrombin, factors VIII, IX and X and antithrombin increased more in group A than in group B (Table 2).
Samples of 32 patients were used for whole-blood thromboelastometry (ROTEM). Fibrin clot formation curves for group B, but not for group A, showed an improvement after intervention of all parameters, including the MCF and α-angle (Table 3).

Table 2: Hematological and coagulation parameters of patients pre- and post-intervention.

	Group A (n=21)			Group B (n=22)		
	pre	**post**	**Δ**	**pre**	**post**	**Δ**
Hb (mM)	5.3 (1.3)	5.2 (1.3)	-0.09	5.5 (1.3)	5.5 (1.7)	+0.01
Ht (%)	0.26 (0.06)	0.24 (0.06)	-0.02	0.26 (0.06)	0.27 (0.06)	+0.01
Platelets (10^9/L)	108 (56)	97 (43)	-12	83 (42)	82 (47)	-1
aPTT (s)	96 (68)	45 (19)	-49 *	93 (48)	70 (40)	-24 *#
PT (s)	23 (30)	13 (2)	-9.5	25 (30)	15 (2)	-10
Fibrinogen (g/L)	1.59 (0.97)	1.64 (0.66)	+0.06	1.22 (0.58)	1.79 (0.61)	+0.57 *##
Prothrombin (%)	36.8 (16.8)	52.1 (18.2)	+15.3 *	31.4 (13.5)	36.6 (13.4)	+5.2 *#
Factor VIII (%)	61.4 (40.3)	85.3 (33.9)	+23.9 *	75.9 (39.5)	80.2 (43.3)	+4.3#
Factor IX (%)	50.1 (27.5)	67.4 (26.4)	+17.3 *	52.3 (30.4)	57.8 (27.4)	+5.8
Factor X (%)	35.2 (15.7)	50.3 (17.9)	+15.0 **	32.5 (14.4)	37.8 (13.8)	5.3 *##
Antithrombin (%)	41.8 (13.9)	55.0 (16.8)	+13.1 *	38.9 (12.7)	42.9 (13.7)	4.0 *#

Data are means (SD); mean differences due to intervention are indicated Δ. *p<0.05 *vs.* pre-intervention, #p<0.05, ##p<0.01 *vs.* group A.

Table 3: Thromboelastometry (ROTEM) parameters.

	Group A (*n*=21)			Group B (*n*=22)		
	pre	**post**	**Δ**	**pre**	**post**	**Δ**
ROTEM (whole blood) [$]						
CT (s)	262 (287)	176 (83)	-86	431 (448)	233 (158)	-198 **
CFT (s)	535 (641)	258 (289)	-277	609 (375)	372 (293)	-237 *
MCF (mm)	40.0 (13.6)	43.4 (11.1)	+3.4	32.2 (12.4)	39.9 (14.7)	+7.7 **[#]
Alpha (°)	48.3 (20.2)	56.0 (13.5)	+7.7 *	34.8 (15.6)	46.7 (17.6)	+11.9 **
ROTEM (PRP)						
CT (s)	138 (59)	107 (17)	-31 *	173 (122)	145 (101)	-28 **
CFT (s)	131 (82)	89 (30)	-42 **	145 (102)	110 (62)	-36 *
MCF (mm)	57.1 (12.2)	60.4 (6.1)	+3.3	54.4 (10.6)	59.7 (6.7)	+5.3 **
Alpha (°)	67.0 (10.3)	72.5 (5.7)	+5.5 *	66.8 (11.9)	71.3 (7.3)	+4.5 *
ROTEM (PFP)						
CT (s)	232 (212)	116 (50)	-117 *	216 (245)	141 (152)	-75 **
MCF (mm)	12.6 (8.3)	13.6 (7.7)	+1.0	9.6 (6.0)	14.9 (6.2)	+5.3 **[#]
Alpha (°)	29.3 (30.7)	44.8 (29.8)	+15.5	22.0 (25.7)	51.7 (27.7)	+29.7 **

ROTEM was performed with whole blood, reconstituted PRP or PFP plus phospholipids in samples collected pre- and post-intervention. Data are means (SD). Mean differences due to intervention are indicated by Δ. In all assays, coagulation was triggered with tissue factor. Analyzed curve parameters were clotting time (CT), clot formation time (CFT), maximal clot firmness (MCF) and α-angle (alpha). [$]*N*=16, *$p$*<0.05, **$p$*<0.01 *vs.* pre-intervention, [#]p<0.05 *vs.* group A.

The intervention effect on MCF was significantly higher in group B (Figure 1A). Thromboelastometry (ROTEM) was then performed with plasma samples from all patients in the presence of donor platelets (reconstituted platelet rich plasma - PRP) or PFP plus phospholipid vesicles. The same trends were observed for thromboelastometry curves with either platelets or phospholipid vesicles present (Table 3). The intervention effect on MCF once more was higher in group B (Figure 1B, C), which corresponded to the more increased fibrinogen level in group B plasmas (Figure 1D).

Thrombin generation was measured with the CAT assay, again using reconstituted PRP or PFP with phospholipid vesicles and triggering by 10 pM tissue factor. Of the two key curve parameters, that is, thrombin peak height and endogenous thrombin potential (ETP), the former was most sensitive to the interventions (Table 4). The thrombin peak height after intervention increased more in group A than in group B (Figure 1E, F). In comparison to previously established cut-off values for normal thromboelastography and thrombin generation in PFP,[(9)] at least one of these values was sub-threshold in 15/21 (71%) and 20/22 (91%) of pre-intervention plasmas from groups A and B, respectively.

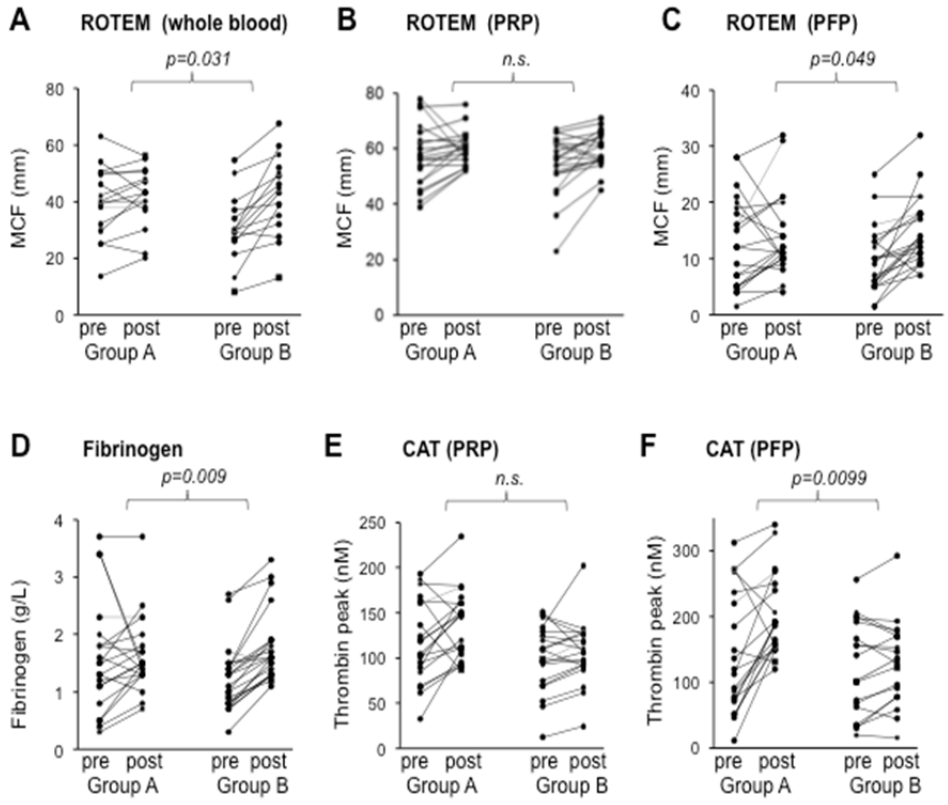

Figure 1: Intervention effects on various coagulation parameters in groups A and B. Data measured in pre- and post-intervention samples are indicated per patient group. MCF of ROTEM in whole-blood (A); MCF in reconstituted PRP (B); MCF in PFP with phospholipids (C); fibrinogen level (D); thrombin peak height of CAT in reconstituted PRP (E) and PFP with phospholipids (F). Significance is shown of difference of treatment effect (Δ) between groups. For mean values, see Tables 3 and 4.

Table 4: Thrombin generation assay (CAT) parameters.

	Group A (*n*=21)			Group B (*n*=22)		
	pre	**post**	**Δ**	**pre**	**post**	**Δ**
CAT (PRP)						
Peak height (nM)	118 (46)	137 (38)	+18 *	99 (35)	111 (40)	+12
ETP (nM×min)	1134 (414)	1193 (358)	+59	1030 (323)	1022 (262)	-8
CAT (PFP)						
Peak height (nM)	142 (92)	200 (63)	+58 *	117 (71)	131 (63)	+14 ##
ETP (nM×min)	993 (431)	1114 (352)	+121 *	899 (322)	886 (243)	-13

CAT was performed with reconstituted PRP or PFP plus phospholipids in samples collected pre- and post-intervention. Coagulation was triggered with tissue factor. Data are means (SD). Mean differences due to intervention are indicated by Δ. Curve parameters analyzed were thrombin peak height and endogenous thrombin potential (ETP). *p<0.05 *vs.* pre-intervention, ##p<0.01 *vs.* group A.

Regression analysis of the data from both groups combined pointed to significant correlations between whole-blood MCF values, on the one hand, and platelet count, fibrinogen level and prothrombin level on the other hand (Figure 2A-C). The MCF of whole blood also correlated with the plasma MCF (Figure 2D), which in turn correlated with the fibrinogen level (Figure 2E). Thrombin peak height in CAT assays furthermore correlated with prothrombin levels (Figure 2F). In all correlation plots, results from groups A and B were equally distributed.

More detailed multiple regression analysis indicated that the correlations between whole-blood MCF and platelet count or fibrinogen level remained significant ($p<0.001$), when the prothrombin level was taken as control variable (*i.e.* as surrogate marker of plasma dilution). However, the correlation between whole-blood MCF and prothrombin level disappeared ($p=0.77$) after normalization for platelet count and fibrinogen level. The MCF in plasma samples correlated with thrombin peak height in the presence ($p=0.009$) or absence ($p=0.027$) of platelets. Thrombin peak height furthermore correlated with most coagulation factors. Together, this indicated that key determinants of whole-blood thromboelastometry measuring fibrin clot formation, are the platelet count and fibrinogen level.

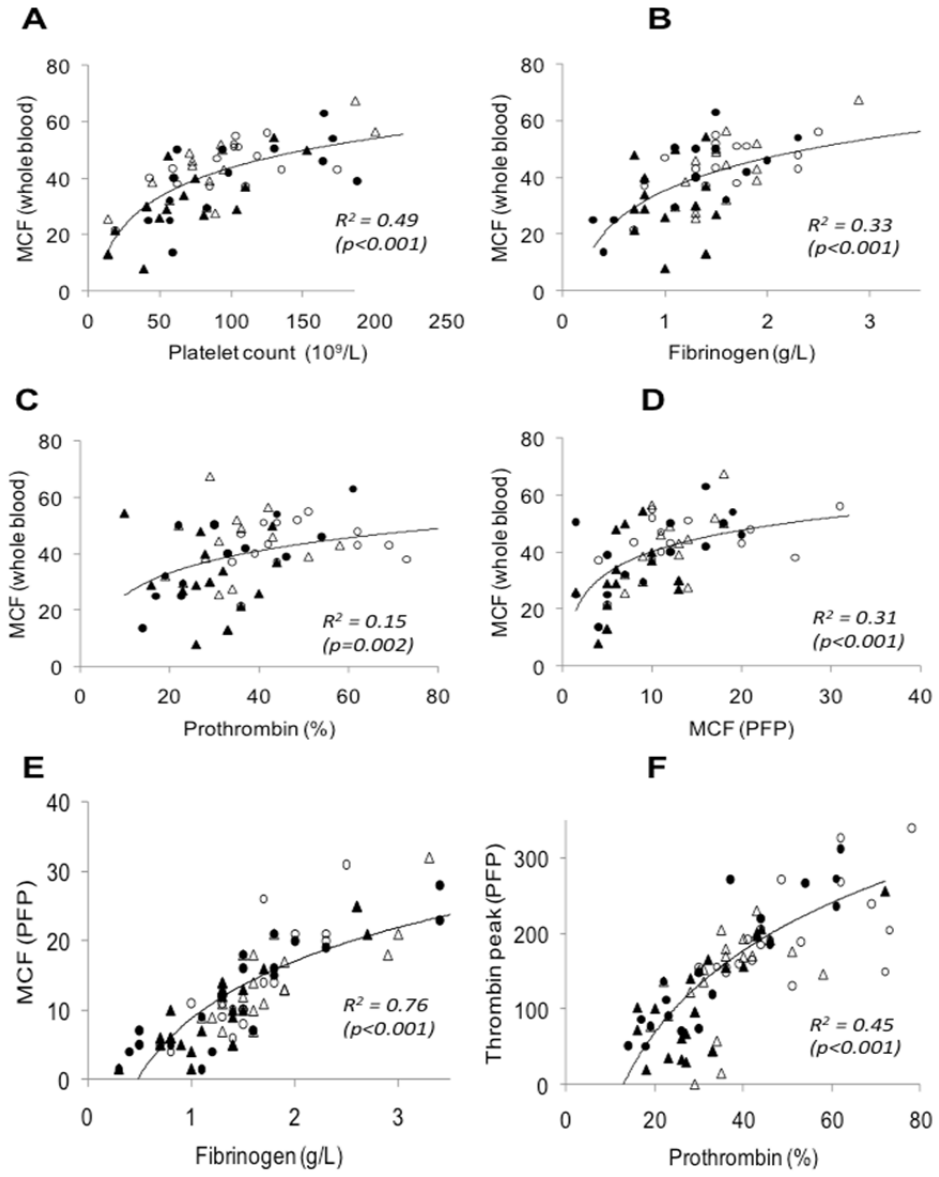

Figure 2: Correlation of thromboelastometry and thrombin generation with various parameters before and after intervention. Regression analysis of whole-blood MCF (ROTEM) versus platelet count (A), fibrinogen level (B), prothrombin level (C), and MCF in plasma (D). Regression analysis of MCF in plasma (ROTEM) with fibrinogen level (E), and of thrombin peak height (CAT) with prothrombin level (F). Data from group A are indicated by bullets, those from group B by triangles (closed = pre-intervention, open = post-intervention).

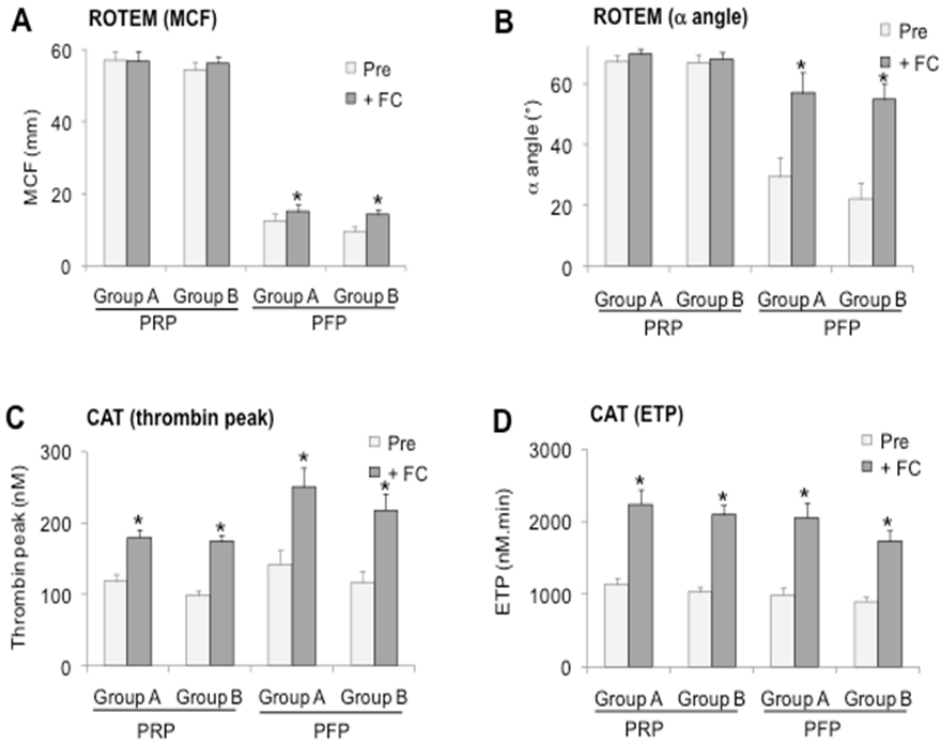

Figure 3: Effects of addition of factor concentrates (FC) on thromboelastometry and thrombin generation. Pre-intervention plasma samples were reconstituted with 0.8 g/L fibrinogen concentrate and/or 0.22 U/mL prothrombin complex concentrate. Thromboelastometry (ROTEM) and thrombin generation (CAT) were measured in reconstituted PRP or PFP with phospholipid vesicles, after triggering with 10 pM tissue factor. Means ± SEM (*n*=20-21), **p*<0.05.

Knowing that in dilutional coagulopathy the low levels of fibrinogen and other coagulation factors result in sub-normal thromboelastometry and thrombin generation,[17] we investigated the effect of *in vitro* supplementation of fibrinogen and prothrombin complex concentrate to pre-intervention patient plasmas. Samples were incubated with both factor concentrates, at amounts corresponding to the expected increase in fibrinogen and prothrombin after transfusion of 4 units FFP, that is, 0.8 g/L fibrinogen concentrate and 0.22 U/mL prothrombin complex concentrate.[9] Because of the limited amounts of plasma presence, *in vitro* addition was only performed with both concentrates together. For thromboelastometry assays in PFP, addition of factor concentrates markedly improved the MCF and α-angle (Figure 3A, B). This corresponded to the intervention effect in groups A and B. In the already high thromboelastometry curves with reconstituted PRP, no extra effect of factor

concentrate addition was seen. On the other hand, in CAT curves with PFP or reconstituted PRP, factor concentrate addition resulted in increased values of both thrombin peak height and ETP (Figure 3C, D), thus resembling the intervention effect on thrombin generation in group A only.

Discussion

This study describes a first prospective, randomized clinical trial, comparing the prohaemostatic effects of partial replacement of FFP transfusion by fibrinogen concentrate in a total of 43 patients with massive haemorrhage. In the patients of group A, receiving 4 units FFP, levels of coagulation factors (with the exception of fibrinogen) were more increased than in group B, which translated to a more improved thrombin generation profile. Conversely, in the patients of group B, who received 2 units FFP plus 2 g fibrinogen, plasma fibrinogen was more increased, which translated into higher thromboelastometry curves. Hence, by partial replacement of FFP by fibrinogen, the improved capacity of fibrin clot formation was accompanied by a reduced normalization of thrombin generation. In group A 16 out of 21 and in group B 17 out of 22 stopped bleeding, while in either treatment arm 5 patients continued to bleed. Limitations of the study were the relatively small sample size with patients undergoing various types of surgery, and the fact that inclusion was based on clinical decision with the attending anaesthesiologist not blinded to the study intervention.

The CAT assay effectively detected a better improvement in coagulant activity, if tested in the absence of platelets, after intervention with 4 units FFP in comparison to 2 units FFP plus 2 g fibrinogen. Comparison of the curve parameters indicated that the thrombin peak height was a more sensitive marker than the ETP. The likely reason for this is that the ETP (area-under-the-curve) is substantially influenced by the calculated level of curve ending, *i.e.* the calculated α2M-macroglobulin correction.[28] Under conditions of hemodilution, this correction may need to be made in a non-standard way. The thrombin peak height, though, correlated well with the plasma levels of prothrombin and other coagulation factors.

On the other hand, transfusion of 2 g fibrinogen plus 2 units FFP resulted in a more active fibrin clot formation, as determined by thromboelastometry (ROTEM) in whole blood and plasma. In agreement with this, we found that, next to the platelet count, the fibrinogen level is the main determining variable of whole-blood

thromboelastometry, which has also been observed by others.[13] In our study, transfusion with only FFP was less effective in increasing plasma fibrinogen than co-transfusion of fibrinogen concentrate. While these data are in agreement with recent surveys,[11, 12] they also give support to the proposal of using a high ratio of FFP to red cells in treatment of massive hemorrhage.[29, 30]

We chose to administer a low dose of 2 g fibrinogen to minimize possible risks for thrombosis. This choice was supported by retrospective investigations, indicating that a single dose of 2 g fibrinogen supplemental to FFP and platelets was effective in resolving hemorrhage.[31] In some clinical centers, fibrinogen is infused at higher amounts up to 14 g to reach plasma concentrations above 1.5-2 g/L.[32] Also in porcine models, higher doses of fibrinogen and prothrombin complex concentrate have been employed to arrest surgical bleeding.[33, 34] Although the two intervention regimes stopped bleeding in the majority of the patients, hemostasis was insufficient in 5 patients of each group. Sub-analysis of the coagulation profile in the patients with persistent bleeding indicated on average low levels of fibrinogen (A: bleeding and non-bleeding, 1.3 ± 0.4 and 1.7 ± 0.4; B: bleeding and non-bleeding, 1.5 ± 0.6 and 1.8 ± 0.6 g/L; mean ± SD) and of prothrombin (A: 43 ± 10 and 55 ±19%; B: 33 ±12 and 36 ± 14%, respectively). Furthermore, the majority of patients with persistent bleeding were low in the previously determined[9] threshold values of normal thrombin peak height (A: 5/5, B: 5/5 patients) and of normal MCF of fibrin clot formation (A: 4/5, B: 3/5 patients). Hence, we inferred that insufficient coagulant activity due to low levels of fibrinogen as well as other coagulation factors was a main cause of persistent bleeding. Hence, the present findings are in support of the earlier conclusion that combined testing of thromboelastometry and thrombin generation can better predict peri-operative bleeding in major surgery than either assay alone.[9]

In conclusion, this trial showed a similar effect of 2 g fibrinogen additive to 2 units FFP compared with transfusion of 4 units FFP on hemostasis. The transfused fibrinogen had an important contribution to fibrin clot formation in thromboelastometry analysis, while the transfused 4 units FFP increased the thrombin generation significantly more but resulted in lower fibrinogen levels compared to the co-transfused fibrinogen. We hence postulate that transfused FFP and fibrinogen are both relevant for early hemostasis and that both should be combined in massive hemorrhage protocols. These results argue for a larger-scale follow-up study, where the effects are determined of supplementing fibrinogen concentrate together with a standard dose of FFP. Herein, a prohemostatic effect of fibrinogen is expected on top of the effect of

normalization of other coagulation factors.

Acknowledgments

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Chapter 9

Summary and General Discussion

Why measure thrombin generation?

Almost 50% of all deaths in western society are caused by cardiovascular diseases.[1] It is therefore of great importance to monitor blood coagulation and the need for new assays capable of detecting abnormal patterns in coagulation in a faster, more accurate and predictive way than by current coagulation tests. Thrombosis and bleeding are limited by the effect of localized and rapid amplification of proteases that are responsible for hemostasis at the site of vascular injury and by the effect of protease inhibitors in the circulation.[2] The capacity to generate thrombin and its enzymatic work determines the coagulation capacity, therefore it is interesting to measure thrombin generation.[2, 3]

The thrombin generation assay is an established research tool in the research field of thrombosis and hemostasis. It mirrors a significant part of the overall function of the blood clotting system. In contrast, the clotting time-based assays that are commonly used do not detect a thrombotic tendency of unknown origin, as well as some mild hemostatic disorders.[3, 4] Through the years several developments made it possible to measure thrombin generation in a high-throughput manner.[3] It has been demonstrated that thrombin generation measured with the Calibrated Automated Thrombogram (CAT) assay, as developed by Hemker and coworkers, is a sensitive tool to detect/predict prothrombotic states (that correlate well with the clinical outcome), bleeding conditions (congenital or acquired) and drug capacity (pro- or anti-coagulant). Depending on the affected part of the coagulation, primary or secondary hemostasis, thrombin generation should be performed in either platelet-poor or -rich plasma as indicated in Table 1.[2, 3]

Table 1: Differences in detecting clotting abnormalities in TG of platelet poor and rich plasma.

TG in platelet poor plasma is sensitive to:	**TG in platelet rich plasma is sensitive to:**
* all clotting factor deficiencies, except for fibrinogen and FXIII * oral anticoagulants: coumarins, heparins, direct thrombin inhibitors, … * hyperthrombinemia, due to lack of antithrombin, protein C, protein S, activated protein C resistance, …	* deficiencies: von Willebrand factor, thrombopenia, Glanzmann, Bernard Soulier * anti-platelet drugs, e.g. aspirin, GPIIb/IIIa antagonists, … * platelet activators: arachidonic acid, epinephrine, collagen, …

Despite the good sensitivity in detecting clotting abnormalities, the CAT assay still has to deal with some issues. Although the variation of this fluorogenic assay is lower than its chromogenic precursor, it still has a substantial inter-laboratory variation.[2] This variation is caused by variability between laboratories in their protocols, software versions, source and concentration of tissue factor (TF) and/or phospholipid vesicles, presence or absence of corn trypsin inhibitor (inhibitor of the contact activation), concentration of the substrate and preheating conditions (as temperature influences the activity of the enzymes). Especially for multicenter studies it is recommended to take precautions by standardization of these contributors that can influence the outcome of the assay. Additionally, it is also recommended to use a reference plasma to normalize the results, as it can reduce the coefficient of variation between laboratories below 7.5%.[2, 5-7]

The applications of thrombin generation

The future for predicting bleeding or thrombosis could be the measurement of thrombin generation.[8] At the moment, the principal cause for the inability to introduce the CAT assay into the clinic is the variation seen in source of the reagents and the variation between laboratories as mentioned above.[2, 6] The clinical utility of a test is to improve the health outcome of patients and blood drawing procedures. For a thrombin generation assay this means that it should have a great accuracy to detect a variable degree of coagulation. The capacity of the CAT assay to detect prothrombotic or bleeding phenotypes has already been accepted for research purposes.[2, 9-27] Data about these coagulation disorders are published by different groups, e.g. thrombin generation in patients with rare inherited coagulation disorders[28], patients with hypercoagulability[29], patients with hemophilia, …[6, 30, 31] Particularly, this assay revealed to be instrumental in the elucidation of the coagulopathy of chronic liver diseases.[32-37] The regular CAT assay can also be modified in order to investigate a specific part of the coagulation cascade.[38, 39] As there was a need for new assays for hemophilia A diagnosis and treatment, we altered the CAT assay such to measure factor (F) VIII levels in the range of 0-2 IU/dL with high precision.[20, 39-42] We investigated the possible correlation between the FVIII level and the bleeding phenotype of severe hemophilia A patients. With our newly developed assay we found that the FVIII level of these patients could not explain the difference in bleeding

phenotype between mild and severe bleeders. When we used the normal thrombin generation assay, we established that the mild bleeding patients might be protected from severe bleedings by factors (other than FVIII) that increase thrombin generation. It still has to be demonstrated in a multicenter study with a large number of mild bleeders whether the thrombin generation assay is useful in predicting the bleeding tendency in an individual. Such an assay is especially interesting for managing prophylaxis in these patients.[39] Further research is needed to detect the factors enhancing thrombin generation that might be associated with the clinical phenotype. It is suggested that a decrease in natural anticoagulants could play a role, e.g. tissue factor pathway inhibitor, protein S or antithrombin.[9, 30, 39, 43]

The CAT assay can also be used to monitor or predict the outcome of transfusion of blood products. Schols et al.[44] showed the effect of *in vivo* transfusion of fresh frozen plasma (FFP) to patients suffering from acquired coagulopathy.[44, 45] We investigated the effect of *in vitro* dilution of blood and plasma on thrombin generation and fibrin clot formation (with thromboelastometry), and by examining how these processes were affected by addition of factor concentrates and blood cells (fibrinogen concentrate, prothrombin complex concentrate, red blood cells and platelets). This *in vitro* study demonstrated that the thrombin generation assay in plasma was insensitive to an increase in fibrinogen concentration, while the thromboelastometry experiments revealed to be sensitive only to changes in platelet and fibrinogen concentration. Addition of prothrombin complex concentrate had a major impact on thrombin generation, but did not affect fibrin formation. It could be concluded that the results obtained with the thrombin generation assay and the fibrin formation assay provide two independent determinants of the bleeding risk of a patient.

This was also confirmed in another study where we investigated the effect of *in vivo* transfusion of FFP with or without fibrinogen concentrate to severe bleeding patients. Partial replacement of FFP with fibrinogen concentrate resulted in an amelioration of the fibrin clot formation, but to the expense of a less improved thrombin generation.[45-47] Since thrombin is a crucial factor that has multiple roles in coagulation and since fibrin formation is the end-point of coagulation, it is better to measure both thrombin generation and fibrin clot formation to have a global and complementary information about the clotting abnormalities of a patient.[45-47]

The CAT assay has also proved to be very useful in fundamental research. We investigated with this assay the effect of the different $\beta_2$glycoprotein I (β_2GPI) conformations on thrombin generation. The physiological role of β_2GPI is unknown.

However, pathophysiologically, β_2GPI appears to be the most important protein against which autoimmune antibodies can develop resulting in the development of the antiphospholipid syndrome.[48-50] In the past contradicting results were published about the role of β_2GPI on coagulation. The recent discovery that β_2GPI can adapt to three different conformations (circular, S-shaped and J-shaped) gave more insights into this protein.[51, 52] We found that addition of the native conformation, which is either circular or S-shaped, to plasma did not affect thrombin generation parameters. Pre-incubation of β_2GPI with an anionic surface (e.g. negatively charged phospholipid vesicles) induced a conformational change from a closed into an open, J-shaped, form of the protein. Addition of this conformation to plasma resulted in a decrease in thrombin generation. This decrease was dependent on the incubation time, the concentration of TF used to activate the sample and the concentration of the β_2GPI that was used.[53] The hypothesis is that after binding to an anionic surface (e.g. the site of vascular damage) β_2GPI changes its conformation thereby revealing part of domain I, which is covered by a carbohydrate chain in the closed native conformation. This part of domain I, the active site that harbors the cryptic epitope G40-R43, was shown to be able to inhibit coagulation (unpublished data). The hypothesis is that autoimmune antibodies in patients suffering from the antiphospholipid syndrome, bind to this cryptic epitope and interfere with the anticoagulant property of β_2GPI causing their prothrombotic tendency.[52, 54-57]

The innovations of thrombin generation

One of the innovations of Synapse was to develop a reliable assay able of measuring thrombin generation in whole blood. In the past other groups tried to develop a comparable assay, however, nobody succeeded to bring it into the lab. Tappenden et al.[58] used a modified CAT assay to measure thrombin generation in whole blood, by the use of the same substrate, Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (ZGGR-AMC) as the regular CAT assay, but measured with a different excitation (390nm) and emission (500nm) wavelength. Regrettably, the imprecision of their assay was rather high, i.e. 14% CV value for the peak height.[58] We tried to repeat their experimental setup but encountered two problems. Firstly, the fluorescent signal was 30x weaker than in plasma, leading to a much higher signal to noise ratio from which the data could not be analyzed. Secondly, thrombin generation curves were higher than

calibration curves, making it impossible to convert the fluorescent data into thrombin concentration.[59] After several prototypes were produced and tested, we developed a CAT-based thrombin generation assay in whole blood, the so-called WB-CAT assay.[60] The problem related to substrate quenching by hemoglobin was partly solved by using a rhodamine-based fluorogenic thrombin substrate. This substrate has a high quantum yield. Moreover, substrate consumption is almost negligible during the time of the assay. Another problem was sedimentation of the red blood cells in time together with clustering and retraction with the formed clot. This was resolved by using a thin layer of a porous matrix that entraps the red blood cells. The WB-CAT assay showed acceptable intra-assay variation of all thrombin parameters. The inter-individual variations of the endogenous thrombin potential (ETP) and thrombin peak height were respectively 23% and 18%, which are comparable to the inter-individual variation found in the plasma CAT (17.5%). Apparently, the presence of blood cells does not significantly add up to a larger individual variation of the whole blood TG parameters. A major advantage of assays using whole blood is that, apart from being closer to physiology, there is no need to centrifuge the blood. The time needed to start the assay will be reduced, as well as the errors than can occur because less laboratory manipulation is required.[60] Thrombin generation in plasma is often conducted with a rather high amount of TF (5 pM) resulting in less variation in ETP and peak height compared to lower TF concentrations. However, an important disadvantage of using a high TF concentration is the loss in sensitivity for the intrinsic pathway. Although the optimal TF concentration for clinical studies has still to be evaluated, we used a low (0.5 pM) TF concentration in the whole blood thrombin generation assay. In that way, the assay remains sensitive to factors of the intrinsic coagulation pathway, as seen by the good correlation between plasma FVIII levels of patients with hemophilia A and the thrombogram parameters ETP and thrombin peak.[60] To avoid interference caused by contact activation in whole blood, we recommend to collect blood samples in a tube containing citrate and a FXIIa inhibitor as earlier suggested for measuring TG in plasma.[60-62]

One major advantage of the WB-CAT assay is the small sample volume needed to perform the assay. This is especially important for mice studies. Mouse models are getting increasingly popular in the research field of thrombosis and hemostasis.[63-67] In mouse plasma Tchaikovski et al.[66] demonstrated with the use of a modified CAT assay hypercoagulability induced by FV-Leiden, oral contraceptives and pregnancy.[63, 66] However, mice have to be sacrificed in order to obtain enough plasma for thrombin

generation experiments. Conversely, using the WB-CAT assay, blood can be taken repeatedly in time from the same mouse and several different conditions can be tested with blood taken from one single mouse. Consequently, the number of mice needed to conduct a study can be reduced.[68]

A disadvantage of the WB-CAT assay is that it requires skills that are available in the research laboratory but which are lacking in routine medical laboratory practice. For this reason we also developed a simple prototype that on basis of the thin layer technology measures the course of thrombin generation in a drop of blood. This prototype can be connected to a smartphone for which we already have developed successfully an application. The advantage is that only a very small volume of blood is needed (15µl), which can be obtained with a finger prick without the need to add an anticoagulant. The simplicity and precision of the method are sufficient to serve as a basis for the development of a dedicated device suitable for the clinical laboratory and/or point-of-care (POC) setting.[69] The POC-TG assay is very promising as it can measure thrombin generation in whole blood, but also in platelet poor and rich plasma. However, calibration is still needed for correcting inner filter effects, substrate consumption and sample color. Another problem is that samples have to be mixed with a mixture of reagents containing an activator and a substrate before they can be applied to the cartridges. These issues make it impossible (for now) to have patients using this POC prototype at home for self-monitoring. A solution for the reagents mixing could be to dry the reagents on the paper, so that after the finger prick, patients only need to put the drop of blood on the paper. Once these problems are solved (calibration and reagent mixing) a good technical and clinical validation has to be done before the thrombin generation-POC can be incorporated into the clinic or used for self-monitoring at home.

The role of blood cells in coagulation

The role of red blood cells in coagulation has recently gained more interest. First, it was thought that red blood cells were inert bystanders of thrombus formation and only played a role in blood viscosity and rheology by “pushing” the platelets against the vessel wall. Nowadays, more evidence has been gained about their contribution to coagulation by exposure of phosphatidylserine (PS) on their outer membrane.[70-72] It has been shown that in healthy people around 0.5% of the red blood cells are PS

positive.[73-76] Apart from the influence on thrombin generation, PS exposure makes the red blood cells more adhesive to the endothelial cells, making them also more prone to form aggregates in conditions like diabetes mellitus, obesity, hereditary stomatocytosis, sepsis or chronic renal failure.[77-80] Barr et al.[81] showed that after $FeCl_3$-treatment of an endothelial surface, red blood cells (and red blood cell-derived materials) are the first to be present at the site of the injury. Afterwards, platelets will be recruited and thrombosis is initiated. The interaction of the red blood cells with the endothelial surface is transient, the red blood cells elongate in the direction of the blood flow and most of these elongated cells break free from their attachment points, leaving small fragments behind.[81] Additionally, blebbing and microparticle shedding often occurs on the surface of the red blood cell.[82, 83] These microparticles expose PS on their surface and can also contribute to coagulation by enhancing thrombin generation in a FXI- or FXII-dependent manner (even in the absence of TF).[71, 74, 82, 84-86] Thrombin generation with the WB-CAT assay in platelet free plasma containing different amounts of washed red blood cells, revealed an increasing thrombin peak height with increasing hematocrit up to 14%. Above a hematocrit of 14% no difference could be observed between activation with and without the addition of exogenous phospholipid vesicles, indicating the contributing role of the red blood cells to thrombin generation. Presence or absence of platelets did not alter thrombin generation. Activation of platelets with convulxin, a snake venom that activates platelets via GPVI, did affect thrombin generation in platelet rich plasma, but not in whole blood.[60] These results are compatible with the findings of Horne et al.[87], that thrombin generation in non-flowing blood is more sensitive to differences in red blood cell concentration than to the platelet concentration.

The role of platelets in plasma coagulation is well known as mentioned in chapter one of this thesis. Platelets can partly compensate for the loss of coagulation factors as recently established.[88] When we investigated the dilution effect of plasma in the presence and absence of platelets, we have seen that thrombin generation and tromboelastography measurements were more affected in diluted samples in the absence compared to the presence of platelets. In thromboelastography we have seen that the platelet count was the major contributing factor to this assay. Only in severely diluted patients with a lower platelet count, the thromboelastography assay became sensitive to the fibrinogen concentration.[44-47, 88, 89] Taken together, these findings suggest that the red blood cells and the platelets interact with each other in the coagulation process. While the red blood cells seem to be more important for PS

exposure (and therefore accelerate thrombin generation in whole blood), the amount of platelets is more important for the strength of the forming clot (thromboelastography) as showed in Figure 1.[45, 46, 71, 74, 88]

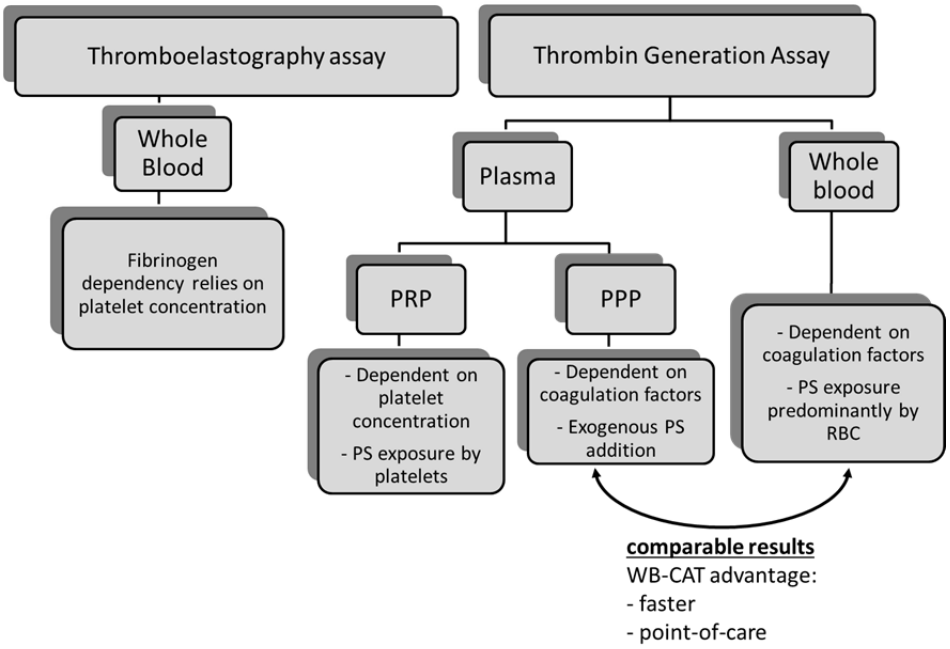

Figure 1: Dependency of platelets, red blood cells, coagulation factors and PS exposure in thromboelastography and thrombin generation assays. Both assays are independent of each other but give complementary information about the global hemostatic status of patient. PRP, platelet rich plasma; PPP, platelet poor plasma; PS, phosphatidylserine; RBC, red blood cells.

Concluding remarks and future perspectives

In conclusion, still a lot of work has to be done before the whole blood thrombin generation assay can be implemented in the clinic. The potential utility of the POC-TG in clinical settings needs to be further investigated and demonstrated. The final, ideal thrombin generation assay is a robust and standardized assay with an acceptable intra- and inter-laboratory variation that includes all blood cells (platelets, red and white blood cells), the vessel wall and flow conditions. Part of the vessel wall can already be included by the addition of TF and thrombomodulin, however for flow conditions, a

new assay has to be developed.[4] At Synapse, we are currently working on a device that can simultaneously measure thrombin generation and fibrin formation under flow conditions in either plasma or whole blood. The flow rate can be adjusted to mimic arterial and venous flow velocities giving the opportunity to investigate its effect on thrombin generation and fibrin formation. The method proved to be sensitive to changes in fibrinogen content and other factor deficiencies, as well as inhibitors of the coagulation cascade. Finally, the assay is very promising in bringing the thrombin generation assay again one step closer to physiology.[90]

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Nederlandse samenvatting

Dit proefschrift beschrijft het onderzoek naar vernieuwingen op het gebied van trombinevorming en de klinische toepassing van deze assay. In **Hoofdstuk 1** worden de verschillende testen beschreven die heden ten dage in het ziekenhuis gebruikt worden om de stolling te onderzoeken. Dit betreft niet alleen de routinetesten die stoltijden meten en hiermee de concentratie aan stollingsfactoren bepalen, maar ook de testen die de plaatjes-functie en -activatie evalueren. Een belangrijke innovatie op het gebied van plaatjesfunctie is ongetwijfeld tromboelastografie. Deze test wordt in het ziekenhuis steeds meer geïntegreerd en gebruikt om patiënten (peri-operatief) te behandelen. De Calibrated Automated Thrombogram (CAT) assay, dat trombinevorming meet, wordt momenteel nog niet als standaardtest in het ziekenhuis gebruikt, maar het is een veelbelovende test die al veelvuldig gebruikt wordt voor onderzoeksdoeleinden.

In **Hoofdstuk 2** wordt de nieuwe volbloedtrombinevorming (VB-TV) test beschreven. Deze assay werd ontwikkeld door de al bestaande CAT-assay zo aan te passen dat deze ook werkt voor volbloed. De belangrijkste aanpassing was het creëren van een dunne laag bloed door een rond filterpapier te gebruiken dat in een 96-wellsplaat past. Hierbij komen de erytrocyten vast te zitten in het filterpapier en kunnen daardoor niet sedimenteren en samenclusteren in het gevormde stolsel. Uitdroging wordt voorkomen door het filterpapier met een druppel olie af te dekken. Een andere aanpassing was het gebruik van een Rhodamine substraat in plaats van het amino-methylcoumarine (AMC) substraat dat in de CAT-assay gehanteerd wordt. Hemoglobine interfereert veel minder met het excitatie- en emissie-spectrum van Rhodamine dan met dat van AMC. De VB-TV assay is reproduceerbaar en in staat om stollingsstoornissen te detecteren. Deze test heeft het principiële voordeel dat trombinevorming wordt gemeten onder omstandigheden die de toestand in het lichaam beter benaderen aangezien ook alle bloedcellen aanwezig zijn. Een praktisch voordeel is dat er niet gecentrifugeerd hoeft te worden, hetgeen tijd bespaart alsook de kans op het maken van fouten. Een ander groot voordeel van de VB-TV assay is het kleine volume bloed dat nodig is (60 µl). Dit maakt deze assay interessant voor het meten van de trombinevorming in proefdieren waar de hoeveelheid bloed gering is, zoals in muizen. Dit wordt in **Hoofdstuk 3** verder uitgewerkt tot een test waarvan de experimentele variatie aanvaardbaar is (<10%). Wij laten zien dat deze test capabel is om stoornissen in de stolling te detecteren aan de hand van experimenten met *Brain and muscle ARNT-like protein (*Bmal1)-knock out (KO) muizen in vergelijking met wild-

type (WT) muizen. Het is alom bekend dat deze KO muizen een versneld verouderingsproces hebben en daardoor een grotere kans hebben op het ontwikkelen van trombose. Met onze test hebben we inderdaad een hogere trombinevorming in de Bmal1-KO muizen gemeten dan in hun WT leeftijdsgenoten. Het feit dat er een kleine hoeveelheid bloed nodig is, maakt dat je de muis niet hoeft op te offeren waardoor er veel meer condities kunnen worden getest met het bloed van eenzelfde muis en de stollingsstatus van een muis in de tijd gevolgd kan worden.

Hoofdstuk 4 beschrijft het onderzoek naar het effect van hypoxie op de stolling. Voor dit onderzoek zijn we met twee groepen van elk 15 proefpersonen naar het Mont Blanc gebied getrokken. Één groep is actief klimmend omhoog gegaan, terwijl de andere groep dat passief via de kabelbaan deed. De reden hiervoor was om het effect van inspanning op de bloedstolling te kunnen onderscheiden van het effect van hypoxie op de bloedstolling. Bloedstalen werden genomen op 50m, 1100m, 2045m, 3100m en 3900m hoogte. Trombinevorming in plasma was hoger in de actieve groep dan in de passieve groep, wat kan verklaard worden door de verhoging in factor VIII als gevolg van een verhoging in Von Willebrand Factor, hoogswaarschijnlijk geïnduceerd door de fysieke inspanning. Trombinevorming in volbloed daarentegen, verhoogde met toenemende hoogte in beide groepen. Uit deze studie kan geconcludeerd worden dat hypoxie de bloedcellen, of de interactie van het plasma met de bloedcellen beïnvloedt, waardoor het een protrombotisch fenotype induceert.

In **Hoofdstuk 5** wordt aangetoond hoe de CAT assay klinisch toegepast kan worden. Het is bekend dat hemofilie A patiënten met een factor VIII gehalte <5% onderling sterk verschillen m.b.t hun bloedingsneiging. Uit de literatuur is ook bekend dat er geen goede correlatie is met het factor VIII gehalte. Dit zou kunnen komen doordat de bestaande factor VIII metingen in het gebied <2% onnauwkeurig zijn. Wij hebben daarom een nieuwe assay ontwikkeld die in staat is om accuraat de concentratie aan factor VIII te kunnen meten onder de 2%. De hypothese was dat hemofilie patiënten met een milde bloedingsneiging nog genoeg factor VIII hebben dat hun beschermt tegen het ontwikkelen van ernstige bloedingen. Uit dit onderzoek bleek dat het factor VIII gehalte van de mild en ernstig bloedende patiënten niet verschilden. De patiënten bleken echter wel te verschillen als de trombinevorming in plasma gemeten werd met de klassieke CAT-methode geïnitieerd met weefselfactor. Dit betekent dus dat bloedende en niet bloedende hemofilie patiënten niet zozeer verschillen in de

hoeveelheid factor VIII die hen rest, maar wel in het gebruik dat ze kunnen maken van deze sporen factor VIII door verschillen in de efficiëntie van de rest van het stollingssysteem. Verder onderzoek is nodig om uit te wijzen wat het mechanisme is en hoe we deze resultaten kunnen gebruiken in de kliniek.

Hoofdstuk 6 beschrijft het onderzoek naar het effect van verschillende conformaties van β_2-glycoproteïne I op de trombinevorming in een plasma omgeving. Hierbij hebben we kunnen concluderen dat de natuurlijke vorm van het proteïne geen effect heeft op de trombinevorming, maar dat de geactiveerde "open" conformatie van de proteïne voor een verlaging van de trombinevorming zorgt. Deze verlaging veroorzaakt door "open" β_2-glycoproteïne I was afhankelijk van de dosis en de incubatietijd. Het feit dat deze verlaging in trombinevorming enkel waarneembaar was bij lage concentraties aan weefselfactor maakt dat β_2-glycoproteïne I waarschijnlijk een effect heeft op de intrisieke stollingsroute van de stollingscascade. Verder onderzoek is nodig om het mechanisme te achterhalen dat hiervoor verantwoordelijk is.

Een andere klinische toepassing van de CAT assay wordt beschreven in **Hoofdstuk 7.** In dit hoofdstuk wordt er gekeken naar het effect van *in vitro* bloed- en plasmaverdunning op trombinevorming en tromboelastografie. De rol van de bloedcellen en de toevoeging van geconcentreerde stollingsfactoren werd ook hierbij onderzocht. We hebben gevonden dat de trombocyten een deel van de verdunning kunnen compenseren, terwijl de erytrocyten helemaal geen effect op de maximale sterkte van het stolsel hadden. Die maximale sterkte blijkt bij lage verdunningen volledig afhankelijk te zijn van het trombocytenaantal. Pas wanneer dit sterk verlaagd is, begint de stolselvorming beïnvloed te worden door de fibrinogeenconcentratie. Toevoeging van fibrinogeen aan sterk verdund plasma had enkel een effect op de sterkte van het stolsel en niet op de trombinevorming. Daarentegen had toevoeging van protrombine-complex-concentraat (bestaande uit geconcentreerd factor VII, IX, X, II, proteïne C en S) aan sterk verdund plasma alleen een invloed op de trombinevorming en niet op de stolselvorming. Deze *in vitro* data werden bevestigd door trombinevorming en tromboelastografie te onderzoeken in bloedstalen voor en na cardiothoracale chirurgie. Na de operatie blijkt het boed van deze patiënten sterk verdund te zijn (±45%), waarbij de effecten in trombocyten-arm plasma op de trombinevorming en tromboelastografie ernstiger zijn dan in trombocyten-rijk plasma, wat de compenserende rol van de trombocyten weer aangeeft. Het effect van *in vivo*

toediening van fibrinogeenconcentraten aan patiënten met sterke bloedverdunning werd beschreven in **Hoofdstuk 8**. Bij dit onderzoek werden ernstig bloedende patiënten willekeurig verdeeld in twee groepen. Eén groep kreeg 4 plasma eenheden en de andere groep kreeg 2 plasma-eenheden samen met 2 gram fibrinogeen. Bloedstalen werden afgenomen voor en na de transfusie en hierin werd de trombinevorming gemeten (met de CAT) en de stolselsterkte bepaald (met de tromboelastografie). Uit deze studie kan geconcludeerd worden dat patiënten die behandeld werden met de fibrinogeen concentraten een verbeterde stolselsterkte vertoonden, maar wel ten koste van een trombinevorming die minder snel steeg dan die bij de patiënten die enkel behandeld werden met plasma.

De samenvattende conclusie van de thesis komt in het laatste **Hoofdstuk 9** aan bod. Hierbij worden eerst de mogelijkheden en de problemen rond de CAT assay bediscussieerd, samen met de innovaties en de klinische toepassingen, inclusief de rol van de bloedcellen hierin. Vervolgens worden richtlijnen gegeven om te komen tot een robuste en gestandaardiseerde trombinevormingtest met een geringe intra- en inter-laboratoriumvariatie. De ideale stollingsfunctietest zou naast de thrombinevorming ook de functie van de vaatwand moeten kunnen testen en dit onder verschillende flow-condities (overeenkomend met veneuze en arteriële stroming). In onze laboratoria is er een apparaat in ontwikkeling dat de trombinegeneratie en de stolselsterkte kan meten onder flow, maar verder onderzoek is nodig om te bepalen of deze veelbelovende assay in staat is om de trombinegeneratie test nog een stap dichter bij de fysiologie van de mens te brengen.

Publications

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Oral presentations and awards:

- ISTH, Amsterdam, 2013. Whole blood thrombin generation in aging mice
- ISTH, Amsterdam, 2013. Conformation of β_2-glycoprotein I and its effect on coagulation. **Young Investigator Award.**
- NVTH, Koudekerke, 2013. The role of different β_2-glycoprotein I conformations and their effect on coagulation.
- Thrombin Generation Summer School, Maastricht, 2012. Whole blood thrombin generation monitored with a calibrated automated thrombogram-based assay.
- CTMM annual meeting, Utrecht, 2012. Whole blood thrombin generation.
- ISTH, Kyoto, 2011. Use of a calibrated automated thrombin generation assay to quantify accurately factor VIII levels in severe and moderate haemophilia A.
- ISTH, Kyoto, 2011. Erythrocyte-dependent thrombin generation in whole blood. **Young Investigator Award.**

Curriculum Vitae

Marisa Ninivaggi was born on January 30th 1986 in Genk (Belgium). She completed in 2004 her secondary education at the Heilig-Hart College in Maasmechelen (Belgium). In 2008 she obtained her Bachelor degree in Biomedical Sciences and in 2010 her Master degree in Clinical Molecular Sciences with honor, at the University of Hasselt (Belgium). She started working in 2010 as a PhD student at Synapse b.v., which is part of the Cardiovascular Research Institute Maastricht (CARIM) of the Maastricht University. She did her PhD under the supervision of Dr. T. Lindhout, Dr. B. de Laat, Prof. Dr. H.C. Hemker and Prof. Dr. ten Cate. During her PhD project she studied the innovations and the different applications of the Calibrated Automated Thrombogram (CAT) assay. She gave oral presentations on several congresses and won the Young Investigator Award twice. She also had the opportunity to visit and collaborate with different researchers abroad: Dr. Yesim Dargaud in Lyon (France), Prof. Dr. Armando Tripodi in Milan (Italy), Prof. Dr. Zaverio Ruggeri in San Diego (California) and Prof. Dr. Marc Hoylaerts in Leuven (Belgium). Currently she is working as a Postdoc within Synapse.

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