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Thrombinoscopy Revisited

Martijn Moorlag

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Thrombinoscopy Revisited

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Contents

Chapter 1

General introduction

Preface

In the assessment of the blood coagulation system, researchers have an ever-expanding toolkit at their disposal. They (re-)design assays to investigate coagulation e.g. under the influence of blood flow[1,2], with and without blood platelets[3,4] in whole blood and/or in the presence of endothelial cells[5]. On the contrary, clinicians are not exactly spoiled for choice, since most of their assays being based on the same clotting time measurements that date back to the middle of the previous century. In other words, there is a need for the clinical implementation of more insightful blood coagulation assays and judging from the number of assays used in research settings there should be sufficient choice.

This thesis focusses on one test in particular, the thrombin generation test, which was first presented by Hemker[3]. The test has been around for over 15 years now and despite its potential as a clinically relevant measurement, still has not been implemented in the clinic. One of the main reasons that halted further clinical development was the high inter-laboratory variability that has been reported over the years[6].

The initial goal of this work was to create a possibility for the clinical implementation of the thrombin generation assay. The experience gained from this work led us to take a step back and identify the sources of variability in the thrombin generation assay, to use this knowledge for the proposition of solutions and to integrate this in the design of a method which is robust enough for clinical implementation.

The coagulation system

This text is aimed at giving an overview of the coagulation system within which new insights can be incorporated. The description leaves room for the ambiguities of the coagulation system where many of its components have multiple, and sometimes contradictory functions in maintaining the dynamic balance of haemostasis.

Ever since the first description of pathological haemostasis by Huang Ti in approximately 2650 B.C[7], the coagulation system and many of its intricacies and interactions have been the topic of (scientific) research. Despite this early observation, it was not until a couple of thousand years later that a more deliberate investigation led to further insight in the formation of venous thrombi. One of the earliest aetiologies stems from Wiseman (1686) with his description of venous occlusion by blood clots through both stasis and hypercoagulability[8].

Throughout the last decades our understanding of the system and the interactions of its constituents has become broader and broader, thanks to advances in technology facilitating more sophisticated approaches.

It is now known that the blood coagulation system consists of a series of zymogens and their pro/cofactors, most of which circulate in an inactive form. Thrombin (FIIa) is the central enzyme within this system[9]. It is, amongst other things, responsible for the cleavage of fibrinogen to fibrin, the main constituent of a blood clot. Thrombin is formed after the cleavage of its zymogen prothrombin (FII). The level of conservation of thrombin throughout evolution highlights the importance of prothrombin in coagulation and ultimately in survival. The sequence identity between human and hagfish (one of the oldest ancestors still in existence, dating back some 300 million years) is 53%[10].

Prothrombin is converted into thrombin after it has been cleaved at two different sites[11]. At the onset of coagulation only a small amount of thrombin is needed to form a blood clot. These trace amounts of thrombin assist in the formation of larger amounts of thrombin via several feedback mechanisms by which it enhances its own propagation. Besides stimulating its own formation, thrombin also activates its own inhibitors. These inhibitors put a halt to the formation of thrombin, ensuring containment of the process of coagulation.

There are several ways in which coagulation can be initiated e.g. by a rupture of an atherosclerotic plaque or by a wound that ruptures a vessel[12]. When the vessel wall is damaged, it exposes the blood to TF, a protein bound to the membrane of perivascular cells. This prompts circulating factor VII (FVII) to bind the membrane

adjacent the now exposed TF. In circulation FVII already has some activity, but when it interacts with TF this activity is greatly intensified[13]. Through this interaction FVII is now able to activate the proenzyme factor X (FX). Activated FX (FXa) in turn, is able to fully activate FVII into FVIIa hereby speeding up its own formation even more. These feedback reactions lead to an explosion of active proteins.

FXa is able to convert prothrombin into meizothrombin, which is an active form of thrombin that remains membrane bound[14,15]. Being membrane bound, meizothrombin is in a perfect position to activate co-factor V (FV) that binds the membrane as well. Activated FV (FVa) forms a complex with FXa and calcium on a phospholipid surface[16] The formation of this so called prothrombinase complex increases the activation of prothrombin over a 1000 times compared to FXa alone[17,18]. The accelerated cleavage by the prothrombinase complex leads to a burst of thrombin.

With such a prolific burst of procoagulant activity, it is easily understood that inactivation of the coagulation system is just as crucial in maintaining a haemostatic balance as activation is. The coagulation system is best viewed as a dynamic equilibrium that is allowed to fall to one side in case of e.g. injury, but which is rapidly restored when coagulation is no longer necessary. When FXa circulates freely, it quickly interacts with a protein called tissue factor pathway inhibitor (TFPI). This FXa - TFPI complex is an excellent inhibitor of the TF-VIIa complex[19,20]. Moreover, FVa (when it is not bound to a membrane) is also a potent inhibitor of the TF-VIIa complex[21]. Therefore, FXa and FVa propagate their own generation when bound to a membrane and they become inhibitors of coagulation when in solution.

This very efficient inhibitory mechanism only allows FXa to be active for a brief period of time. Consequently, coagulation would not be able to reach the explosive state under low TF conditions, if it were not for an additional pathway. This pathway named the Josso loop[22] after its discoverer François Josso (1927-1981), is triggered by the FVII-TF complex through the activation of FIX. Activated FIX (FIXa) is able to activate FX when it is bound to a phospholipid membrane and when it is in complex with activated factor VIII (FVIIIa), this FIXa-VIIIa complex is also known as the “tenase complex”[23 24].

Factor VIII (FVIII) is present in plasma bound to von Willebrand factor (VWF). As long as FVIII is bound to VWF it is unable to bind to a phospholipid membrane and form a complex with FIX. Thrombin is necessary to cleave FVIII upon which it loses its affinity for VWF. When FVIIIa is in solution, it can bind to phospholipids[17]. Unlike the activation of FV by meizothrombin, the activation of FVII requires thrombin in

solution, that, unlike meizothrombin, is at risk of being cleared by antithrombin (AT), particularly in the presence of heparin.
The combination of both pro- and anticoagulant characteristics of FXa and FVa, ensures that coagulation takes place where it is necessary: on damaged cells and platelets where the procoagulant membranes are found. The spatial delineation of coagulation is further aided by TM, which is expressed on the surface of intact endothelial cells[25]. Thrombin that is bound to TM is able to cleave protein C (PC) to its activated form (APC), and due to this action thrombin loses its procoagulant properties[26].

APC is a strong inhibitory protein that, with the help of protein S and FV[27,28], is able to degrade FVa and FVIIIa which are bound to procoagulant phospholipid surfaces[27,29]. When the procoagulant surface is provided by platelets APC is not able to effectively cleave FVa[30], in fact platelets seem to protect FVa from cleavage by APC[31]. This specificity has led to the believe that APC is not necessarily responsible for the delimitation of thrombin formation but rather that it is responsible for the prevention of clotting on intact vasculature[32]. Compared to APC protein S has a greater inhibitory potential, as it is able to inhibit the prothrombinase complex on endothelial cells as well as on a platelet surface[33].

APC is cleared from the bloodstream by the serine protease inhibitor (serpin) protein C inhibitor (PCI). PCI is a very indiscriminative inhibitor which has in fact a higher affinity for other coagulation factors e.g. FXa, thrombin and FVIIa-TF than for APC[34].
Fibrin was initially named antithrombin 1[35] because it binds thrombin strongly. It has been shown that fibrin(ogen) is primarily a protector of thrombin activity, because of the limited access of $\alpha_2$macroglobulin (α2-M) to thrombin that is adsorbed to fibrin[36]. The most obvious antithrombin action of fibrin and probably the most teleological "reason" that a clot forms as soon as a trace of thrombin is formed, is that the clot is a spatial delineator. The structure of the clot prevents thrombin being washed from where it is most needed, or to spread in free solution where it can work disasters[37]

The blood platelets play a major role in coagulation in vivo. Due to the complexity of their role, this will not be described in detail here. For a comprehensive review, please refer to the subchapter on platelets in Versteeg et al.[32]. In brief, the role of platelets in coagulation starts when endothelium damage occurs. Upon damage, platelets are triggered to adhere to the wound and to each other. Several mechanisms are responsible for the increased platelet adhesiveness, amongst others: shape change, clustering of receptors and increased receptor expression[32]. Aggregating platelets also actively participate in coagulation. From the moment

they are triggered to aggregate, some platelets are activated and start to express phosphatidylserine on their surface[38,39], thereby providing a procoagulant surface. As was explained earlier, many coagulation proteins bind to such procoagulant phospholipid membrane layers where they react with other coagulation proteins. However, the existence of non-obstructive thrombi in large vessels is an indication that the forming plug, at some point, loses its propagating capabilities. This retardation of the growth of the plug is a phenomenon that is still poorly understood.

Platelets and the coagulation system are closely intertwined and their functions are not those of two separate processes. The group of Bruce and Barbara Furie showed that the formation of fibrin takes place within 15s after a lesion is made in the vessel wall[40]. This rapid formation of fibrin indicates that thrombin must have been present almost at the onset of the formation of the platelet aggregate. This is further exemplified by the fact that it takes platelet poor plasma between 12-15s to clot at optimal TF concentration. Wherever there is fibrin, thrombin must have been present at ~15s before its appearance to provide the feedback reactions for coagulation to accelerate. Their exact role is still the subject of debate, but it has been shown that microparticles do play a role in coagulation as well[41]. It has been argued that they adhere to a site of lesion before platelets and they are instrumental in generating thrombin[42].

Further undermining the paradigm of primary haemostasis by platelets the bolstering of a primary plug by thrombin reinforces the idea that platelet aggregation is one voice in a concert of interactions involving plasma proteins and structured elements. Moreover, platelets play a vital role in the localization of coagulation at the site of injury. The confinement of the platelet aggregate reduces molecular transport rates within its core. This increases the concentration of soluble agonists which accelerate thrombin formation and subsequent platelet activation. Local accumulation of thrombin and the regulation of the density of platelet packing both contribute to thrombus growth. Moreover, the higher transport rates in the more permeable outer shell of the aggregate facilitate clearance and limit thrombin formation[43].

The large amount of thrombin localized in the clot activates thrombin-activatable fibrinolysis inhibitor (TAFI)[26]. TAFI protects degradation of fibrin by cleavage of the C-terminal residue, thereby protecting fibrin from degradation by plasmin[27]. This feedback mechanism leads to the formation of a specific clot morphology, that is dependent on the amount of thrombin in the clot. A lot of thrombin gives a dense network of thin fibrin fibres, whereas a small amount of thrombin gives a coarse network of thick fibres[46,47].

Given the efficiency of all of these procoagulant processes it is not hard to imagine that there are counteracting forces present as well. Several have been mentioned already, but the main custodians are the antithrombins. The antithrombins present themselves to thrombin as a substrate to be cleaved, but after doing so thrombin finds itself to be stuck and incapable of further action[35,48]. The most important of the antithrombins was traditionally called antithrombin III (ATIII), and is responsible for clearance of about 2/3 of the total amount of thrombin[49]. The 2nd most important antithrombin is α2-M that clears about 1/4 of the total amount of thrombin[50], the remaining fraction is cleared by miscellaneous inhibitors. Because there are so many antithrombins, we hesitate to follow the official nomenclature and will speak of "antithrombin III" when referring to the main antithrombin.

The efficiency of ATIII is enhanced a great deal by heparin (a common anticoagulant drug). Heparins are glycosaminoglycans that consist of chains of alternating residues of d-glucosamine and uronic acid, glucuronic acid or iduronic acid[51]. Along the chain of saccharides, every 50-100 sugar units a specific sequence of 5 saccharide units occurs that has the ability to strongly bind ATIII[52], which brings about a conformational change in ATIII that enhances its activity. Thrombin binds loosely anywhere to the saccharide chain without the need for a specific saccharide sequence. It moves quickly along the chain by lateral diffusion and so will meet-up with ATIII more easily, so much more easily in fact that heparin can increase the inhibition rate a thousand fold[53].

Measuring the coagulation system

A concise overview of the coagulation tests used in the clinic is given by Bates and Weitz[54]. Although the paper was published in 2005, the text is still very representative of the current situation in the clinical coagulation laboratory. The overview below gives a concise summary of the work of Bates with a few additions in the case of newly implemented test. The main aim of the representation of the tests below is to stress that these tests and their results do not represent the entirety of the coagulation system.

Most coagulation tests are defective approximations of a (specific) part/function of the coagulation system. They rather emphasize the initiation mechanism instead of the integral system of thrombin production. When rigorously standardized a clotting time, like the prothrombin time (PT) can be quite useful, e.g. in the monitoring of anticoagulant therapy with vitamin K antagonists[55]. The activated partial thromboplastin time (aPTT) defies all standardisation efforts[56] and is unsuitable for the monitoring of heparin therapy[57].

It is their representation of the initiation phase that makes clotting times inept for testing conditions that depend on the production phase of thrombin generation, i.e. thrombotic tendency or bleeding tendency dependent upon the amount of thrombin formed. In order to represent the entirety of the complex system, a test is required that goes beyond the initiation phase. That the amount of thrombin is important follows e.g. from the thrombotic tendency that occurs when the protein S dependent system fails (by the use of oral anticonception or factor V Leiden). Why the amount of thrombin is important may be due to various causes, such as e.g. insufficient defence against fibrinolysis through lack of TAFI activation.

Despite the specific nature of the tests described below, they are at times applied for the detection of other pathologies than those described here. It is not impossible to do so, but it has to be understood that value of the results of an assay that measures the starting mechanism of coagulation only, is very limited in describing the complexity of a system as a whole. Most of the current coagulation tests operate in the same general principle as depicted with the APTT below. The variations can be found in the contents of the test tube with differing reagents or by the use of whole blood or blood plasma.

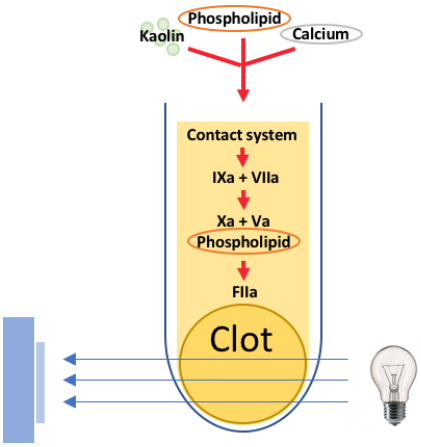

Figure: 1 Activated partial thromboplastin time (APTT).
Sample used: Citrated PPP, *Activator*: FXII activator e.g., kaolin, ellagic acid, celite or silica + diluted phospholipids (cephalin) and calcium, *Used for*: FVIII, FIX, FXI, FXII, HK, prekallikrein, FX, FV, FII, and/or fibrinogen deficiencies and monitoring of intravenous unfractionated heparin.

As can be observed in figure 1, a trigger (in this case a mixture of phospholipids, calcium and kaolin) is added to platelet poor plasma. This sets in motion a series of reactions that ultimately lead to the formation of a clot. A photo sensitive optic, that is able to record the disturbance in the light detects the clot at which moment

the test stops and the time is recorded. As noted before, the tests described below are all clotting time tests and a apply a similar principle but differ in their trigger as well as reaction substrate.

- Prothrombin time (PT), *Sample used*: Citrated PPP, *Activator*: Recombinant or purified TF *Used for*: FII, FV, FVII, FX and fibrinogen deficiencies[54,58]. Vitamin K def. and fibrin degradation products. Warfarin, acenocoumarol, and fenprocoumon therapy through the INR.
- Activated clotting time (ACT), *Sample used:* Whole blood *Activator:* Kaolin, celite or glass particles *Used for*: High dose heparin treatment or bivalirudin treatment. *Notice*: besides systems using optical detection, devices that apply electro-chemical detection of thrombin cleaving an electro-chemical substrate are available on the market as well[59].
- Thrombin clotting time (TCT), *Sample used*: Citrated PPP *Activator*: Thrombin *Used for*: Low fibrinogen, dysfibrinogenemia or elevated fibrinogen degradation products.
- Ecarin clotting time (ECT), *Sample used*: Citrated PPP *Activator*: Purified meizo-thrombin activator from the venom of the Russels viper (*Echis Carinatus) Used for:* Treatment with direct thrombin inhibitors[60].

Apart from these clotting time tests, there are other specific coagulation test that are currently use. Most of these apply different methods of detection making it difficult to cluster them. The "other tests" are usually methods to determine a single element (factor) of the clotting system (Fibrinogen or factors VIII or IX for the management of haemophilia), tests designed for measuring specific anticoagulant treatment (e.g. anti-factor Xa activity for heparins) or indicators of ongoing coagulation (D-dimers, TAT-complexes). They will not be further discussed here.

Most tests that are being used in the clinic have faced the challenge of standardisation. Standardizing tests is crucial to get consistent results but has proven to be difficult for any clinical test and coagulation tests are no exception to this rule. One of the main contributors to the high inter-laboratory variability, of what can be considered a "simple" coagulation test like e.g. a clotting time, has been the composition of the reagents. In an attempt to standardize the PT the International Normalized Ratio (INR) has been established by the world health organization (WHO)[61]. Without this effort of standardization, PT results using different reagents cannot be compared[62]. However, despite the stringent standards, unreliable reporting of the international sensitivity index (ISI) by thromboplastin manufacturers[63] and the variation in precision of INR determination as a result of different reagent and coagulometer combinations still causes variation of the results between laboratories.

Most research laboratories, do not have to abide to the stringent clinical requirements. Therefore, researchers have much more tools at its disposal to measure the coagulation system. Coagulation tests in the research laboratory range from specific test for platelet function to rheology or flow measurements. The present work however is restricted to the thrombin generation test. Thrombin being responsible for the "final" step of the coagulation system with the cleavage of fibrinogen to fibrin is dependent upon the interactions of the preceding proteins for its formation. Therefore, measuring thrombin formation over time gives an indication of the functioning of the system as a whole, way beyond the formation of a clot which already appears when only ~5% of all thrombin is formed. This broader view generated by the TG test gives it the potential to provide qualitative and quantitative information about the coagulation system as a whole.

Throughout the last decades considerable evidence has been published that the amount of thrombin activity that develops in a sample of clotting blood(-plasma) is an indicator of the risk of bleeding[64–67] as well as venous thrombosis[68–76]. Moreover, it relates to the risk of arterial thrombosis, be it in a less straightforward manner[77–79]. It is a common denominator of the effect of all anticoagulants[80–87] and, when it is measured in platelet rich plasma, it is inhibited by platelet inhibitors[88,89]. Therefore, it has a promising outlook for use as a clinically useful parameter.

Similarly to the more basic clotting time based assays, the Calibrated Automated Thrombin Generation test (CAT-TG)[3] that has been used in research laboratories for over 15 years now, has its share of difficulties with respect to standardization and reproducibility of the results between different laboratories.

This poor inter-laboratory variability is considered to be the main issue preventing broad clinical implementation of TG-test[6]. The high variability in test results between laboratories has been attributed to e.g. a lack of automation and its sensitivity to pre-analytical variables[90]. Rigorous standardization of the pre-analytical and analytical procedure and normalization relative to a reference plasma indeed improved the CV[91,92]. Moreover, a multicentre study from French-speaking countries (France, Belgium, Switzerland) carried out under "real life" conditions in clinical and research laboratories showed that the use of a common reference plasma could improve inter-laboratory variability of most TG parameters except in cases of frank hypo-coagulability [93]. These efforts have as yet not added up to a test which is sufficiently robust to warrant broad acceptation in the clinical laboratory.

Measuring thrombin generation

Thrombin generation (TG) was first described in 1953 by MacFarlane and Biggs as a whole blood test[94]. In the same edition of the same journal Pitney and Dacie described the test in recalcified plasma[95]. Both methods were triggered by adding preheated calcium to a sample of clotting blood or plasma, out of which, individual timed (sub)samples were then taken at regular intervals. The subsample then is transferred to a test-tube with a fibrinogen preparation and the clotting time is recorded. The relation between clotting time and thrombin concentration has been previously established. From the observed clotting times the formation and subsequent decay of thrombin in these samples is then calculated. Despite its labour intensiveness this TG test, sometimes in a personalized version, has proved itself useful in advancing research in e.g. haemophilia[96,97].

Before TG could be more widely adopted, the test had to be fundamentally altered. In the present situation of the clinical chemistry lab, where laboratory work is reduced to putting samples in an automaton and waiting for the figures that it prints out – in this ambiance anything looking remotely as a subsampling experiment is an anachronism. A fundamentally new approach was required. This came in three concurrent steps. The first was from S. Béguin who added a chromogenic thrombin substrate directly to the clotting plasma. The second from C. Hemker, who realized that, if this were to reflect the complete thrombin generation curve, the substrate used should be so poorly reactive to thrombin that it should not be consumed during the course of the thrombin generation, and the third from R. Wagenvoord who suggested that – in order to be able to know what reaction velocity reflects what thrombin concentration, a calibration experiment had to be run in parallel. The combination of these three ideas allowed continuous measurement of thrombin formation[98]. In this way, through the detection of the reaction product that is cleaved from this substrate by thrombin, the formation and subsequent decay of thrombin in a plasma sample can be measured over time. Because the formation of fibrin disturbs the detection of the reaction product, the plasma has to be defibrinated prior to the measurement, or a fibrin polymerization inhibitor had to be added.

Fibrin, however, is an essential part of the thrombin generation mechanism[36] so it was an essential step forward when the fluorogenic substrates brought an end to this inconvenience. This led to the presentation of Calibrated Automated Thrombography (CAT)[3].

The idea of using a fluorogenic substrate not being patented it could be followed in the Technothrombin TGA method[99]. The simultaneous calibration through a parallel experiment had been patented, however, so that accurate calibration in the latter method will have to wait for the patent to expire.

The use of both chromogenic and fluorogenic substrates has led to a bifurcation in the commercially available thrombin generation assays. Currently there are three automated TG assays available, two that use the fluorogenic substrate: ST-Genesia by Diagnostica Stago, Inc. (Asnieres sur Seine, France), the CEVERON alpha TGA by Technoclone Herstellung von Diagnostika und Arzneimitteln GmbH (Vienna, Austria) and the Behring Coagulation System (BCS) Siemens Healthcare GmbH (Erlangen, Germany), using a chromogenic substrate.

Because of the important role of fibrin in physiological thrombin generation we restricted our research to methods using a fluorogenic substrate. The article by Kintigh et al[99] is referred to for a comparison between the three automated assays. Besides the three automated methods for TG, four semi-automated TG assays exist. The same bifurcation as with the automated methods persists here with two assays using a chromogenic substrate and two using a fluorogenic substrate. The assays using a fluorogenic substrate can be seen as predecessors of the ST-Genesia and the Ceveron alpha TGA, as this method of TG is the further focus of this work they will be elaborately discussed below.

But first, a brief review of the two assays that make use of the chromogenic substrate: HemoScan Thrombin Generation Assay (HemoScan, Groningen, The Netherlands), is a kit that is intended to measure the influence of biomaterials on thrombin generation. A plasma sample is exposed to the biomaterial to be investigated, where after the TG results with and without exposure are compared. The method utilizes subsampling, rather than continuous detection. After the sample is taken from the reaction mixture, the reaction is stopped and the formed thrombin is left to react with a chromogenic substrate. The thrombin concentrations in the subsamples are subsequently determined from a calibration curve. The HemoScan assay serves its purpose but is not intended for diagnostic use.

Pefakit in-TDT (Pentapharm, Aesch, Switzerland), triggers thrombin generation by contact activation. The cleavage of a chromogenic substrate is subsequently detected over time by measuring the optical density of the reaction mixture. A fibrin polymerization inhibitor is added so as to prevent the formation of a fibrin clot and the related optical disturbance of the measurement. After taking the first derivative of the data this method determines two parameters: the lag-time and the peak. The results are not calibrated against a standard concentration of thrombin, rather the values for the patient sample are expressed as a percentage of normal.

The CAT assay measures thrombin generation in a sample of platelet poor plasma (PPP) or platelet rich plasma (PRP)[3] and has been further developed to be used in whole blood[100]. The TG experiment is based on the detection of the reaction product of a ZGGR-AMC substrate by thrombin. The amount of thrombin in the sample in which thrombin is generating (the staple sample), is calculated by comparison to the fluorescence that develops in a separate sample of the same plasma in which a constant known amount of thrombin activity converts the substrate (the calibrator sample). In this way, the value of the calibration factor can be used to calculate the concentration of thrombin. How this is done and how it should be done is the main subject of this thesis. Figure 1a displays a typical fluorescence trace of such experiment. The speed with which thrombin is formed over time (i.e. the first derivative of the original curve) gives a rough approximation of the thrombin generation curve (figure 1b) and allows to distinguish the separate phases of the process: the lag-time (1), the burst of thrombin formation (2) and thrombin decay (3). It can also be noticed is that the curve does not return to the x-axis due to residual thrombin activity of the α2-macroglobulin thrombin (α2M-T) complex. Thrombin entrapped in the α2macroglobulin molecule loses all known physiological activity but still can split small substrates. This phenomenon and how to disentangle it from real thrombin activity will be discussed in more detail in following chapters.

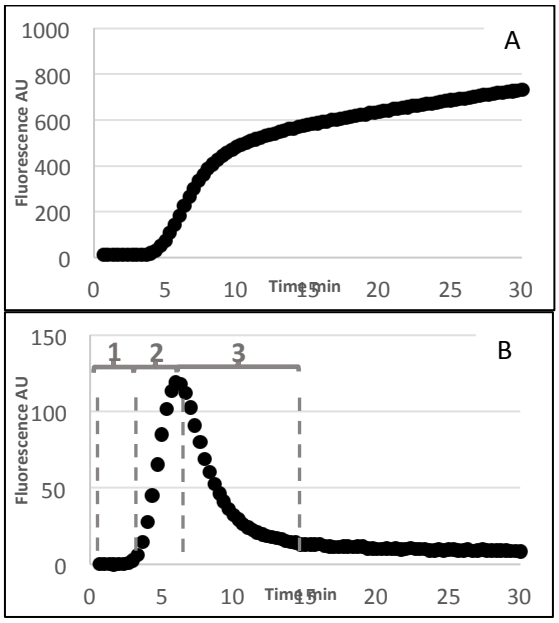

Figure 2 thrombin generation data.
A, "raw" fluorescence trace of thrombin generation. B, First derivative of A with lag-time "1", thrombin formation "2", and thrombin decay "3".

The reaction velocity during the experiment is not determined by the concentration of thrombin (and (α2M-T) alone, also the concentration of substrate plays a role here. Because during the reaction the substrate is consumed the same amount of thrombin will not cause the same reaction velocity in the beginning of the experiment and later on.

Also, the relation between fluorescence and concentration of product is not linear. The consequences of substrate consumption and inner filter effect and how to correct for them will be discussed in much detail in the following chapters. Here we only stress the unique feature of the CAT-method, i.e. that for each TG-experiment there is a parallel calibration experiment in which, in the same plasma, the same amount of substrate is converted by a known fixed amount of thrombin activity (in the form of the (α2M-T complex)[101]. This provides a sample-specific calibration factor at every level of fluorescence and therefore in principle allows correct determination of thrombin concentrations. How to carry out such calculations and searching for alternative but equally correct methods of calibration is the main subject of this thesis.

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Chapter 2

Near Patient Thrombin Generation in Patients Undergoing Elective Cardiac Surgery

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Abstract

Background Measuring thrombin generation (TG) in plasma increasingly gained attention as a diagnostic tool in the field of thrombosis and haemostasis. To include the contribution of all blood cells, recently the whole blood TG method was developed.

Methods We changed the calculation method of the standard calibrated automated thrombography (CAT) to a method only considering the data until the peak of TG, thereby considerably reducing the time from blood draw to result. By redesigning the method, the blood volume per test was reduced to 15 µl.

Results For all TG parameters the inter-assay variation proved to be below 15%. The inter-individual variation of all parameters was comparable to the CAT method. 33 patients undergoing cardiothoracic surgery were included to investigate whether our assay correlates with post-operative blood loss. Upon dividing patients into severe and mild bleeders, significant differences between both groups were found for the peak endogenous thrombin potential (peakETP) and peak values determined by our near patient device. Importantly, patients with a peakETP below the median, experienced significantly more blood loss compared to those with a peakETP above the median. A similar division based on the peak as well as the body mass index of the patient yielded similar significant differences. A combination of the peakETP, the body mass index, and the lag-time even resulted in a better predictor of blood loss compared to each parameter separately.

Conclusions Our adapted whole blood TG assay can be used near patients and is indicative for the amount of blood loss post cardiothoracic surgery.

Introduction

The global haemostatic assay that measures thrombin generation (TG) in plasma increasingly gained attention as a diagnostic tool in the field of thrombosis and haemostasis. TG can be used to distinguish mild from severe bleeding phenotypes in haemophilia A patients with comparable factor VIII levels[1]. Conventional assays, such as the prothrombin time and activated partial thromboplastin time, are based on measuring clotting times. *In vitro* fibrin formation, however, occurs when less than 5% of the total amount of thrombin has been formed[2]. This indicates that the whole haemostatic capacity cannot be adequately evaluated by an endpoint clotting assay[3,4].

The calibrated automated thrombography (CAT)[4] uses platelet-poor (PPP) or platelet-rich (PRP) plasma and a fluorogenic thrombin-sensitive substrate to determine the rate and extent of TG after initiation of coagulation. Coagulation is triggered with tissue factor (TF) (extrinsic pathway) or an intrinsic pathway initiator (e.g. kaolin or ellagic acid). Recently, the CAT-method was modified enabling the measurement of TG in whole blood by using a thin layer methodology[5,6]. By applying TG in whole blood, the assay includes the contribution of blood cells to coagulation. Moreover, avoiding the preparation of PRP or PPP our whole blood approach reduces the assay time and thereby the time it takes to get the patient's result. Another advantage is that the method uses a low volume of blood per experiment[6]. Interestingly, these properties of the novel whole blood method allow the development of a near patient TG test.

The key objective of near patient testing is to have a device that is able to generate results in an acceptable time, without the need of highly skilled laboratory staff[7]. As a result, appropriate treatment could be determined in a shorter time-period improving clinical outcome. Other global tests to study haemostasis (e.g. viscoelastic tests) are increasingly used in the diagnosis and treatment of coagulation abnormalities in critically ill patients[8,9]. In addition, the overall cost of such methods is considerably lower. Current haemostatic techniques for near patient clinical use include whole blood platelet function tests (aggregometry)[10], viscoelastic tests of fibrin formation (thromboelastometry)[11], activated clotting time and International Normalized Ratio measurements. However, each of these assays is limited in its ability to fully evaluate a patient's coagulation profile and hence prediction of bleeding risk or thrombotic complications.

Near patient TG testing has been previously hinted[12–15]. First approaches were based on either an electrochemical principle[14] or fluorescence principle[15]. To the best of our knowledge, none of these methods have been technically and clinically validated. In this paper, we report the development of a miniaturized whole blood

TG test and a quick thrombogram analysis. The clinical potential and practicality of the novel TG assay was tested in a population of patients (n=33) undergoing elective coronary artery bypass grafting (CABG), a vulnerable patient group in which post-surgical bleeding is a frequently occurring complication.

Materials and methods

Healthy controls and patients

After approval of the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Centre) 5 healthy adult volunteers not taking any drugs for at least two weeks gave full informed consent according to the Helsinki declaration. Blood was collected aseptically by antecubital puncture into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System). The blood was kept at room temperature (21°C) and used within 4 hours after withdrawal. Platelet-poor plasma (PPP) was obtained by double centrifugation at 2630g for 10 min and stored at -80°C until further analysis.

The cardiothoracic surgery study was approved by the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Centre) and written informed consent was obtained from all volunteers. In total, 47 patients undergoing surgery for elective CABG were included. Exclusion criteria were age < 18 years and duration of extra-corporeal circulation (ECC) < 60 min. 14 out of the 47 patients were excluded, 12 of which had a duration of the ECC of < 60 min and 2 underwent additional surgical procedures besides the CABG. Blood samples, taken at pre-bypass before heparin administration, were withdrawn from the arterial line after discarding the first 10 ml and collected into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System).

Clinical management

After being connected to standard monitoring (electrocardiography, non-invasive blood pressure, pulsoximetry), all patients received a venous (Vasofix Safety, 16G, 1.7×50 mm) and arterial line (Radial Artery Catheterization set, 20G, 1.1×44.5 mm Arrow International) with 2ml xylocaine 1% as local anaesthesia. Anaesthesia was introduced according to local standards (etomidate, sufentanil and rocuronium in a weight adapted dose) and patients were orally intubated. A large bore central venous line was placed in the right jugular vein and upon clinical need a TEE-probe was inserted for perioperative echocardiography. Anaesthesia was maintained by infusion of propofol 3-4 mg/kg/h and repeated injections of sufentanil boli according to the clinical need. Before initiation of cardiopulmonary bypass (CPB) anticoagulation was achieved by administration of 300-400 IU/Kg unfractionated heparin (Heparin Leo, Leo Pharmaceutical Products BV) to achieve an activated clotting time above 400 s. After termination of the CPB the initial heparin dose was

reversed with protamine in a 1:1 ratio. Components of the CPB system were poly-2-methoxyethylacrylate coated (Terumo). The primer for the CPB circuit consisted of 1300 ml of 4% gelofusin, 200 ml 20% mannitol, 100 ml 20% human albumin, 50 ml 8.4% $NaHCO_3$ and 6500 IU unfractionated heparin. Patients were kept at normothermia (36°C) during CPB. Pericardial, pleural and residual blood of the CPB circuit was drained and washed with a cell saver device at the end of the perfusion. The transfusion trigger was set at a haematocrit below 23%.

Pre-operative data collection included demographics and drug usage. Peri-operatively, medication, infusion volumes, transfusion requirements, time on bypass, cross-clamping time, duration of the surgery, number of grafts, necessity of re-thoracotomy and blood loss were recorded. Post-operatively, the infusion volumes, transfusion requirements and the blood loss were recorded. The latter was assessed by the drainage amount of the chest tubes, which are inserted prior to closing the chest until 24 hours after surgery, when they are removed and the patient is discharged to the medium care (further referred to as "drainage volume").

Haematocrit, platelet counts and fibrinogen concentration measurements

Haematocrit and platelet counts were determined using a coulter counter (Coulter LH750 haematology analyser, Beckman Coulter Inc.). Fibrinogen concentrations were determined using a coagulation analyser according to the manufacturer's instructions (STA-R, Diagnostica Stago).

Miniaturized TG device and microfluidic chip

The measurement module of the device is a dual canal fluorescence detector (ESELog, Qiagen Lake Constance, Germany). Due to its low operating voltage and lack of moving parts, the detector can easily be integrated into various measurement processes. The detector works with impinging light and is equipped with confocal optics. It is connected via a mini to micro-USB cable to an android smartphone equipped with dedicated software (Synapse BV, Maastricht, the Netherlands) for data collection (figure 1A). The detector, holder for microfluidic chip, and temperature control system are placed in a custom housing with dimensions 13×9×6 cm (figure 1A). Temperature (37˚C) control of the chip and its holder is performed with a foil heater, temperature sensor and controller (Minco, by Alflex the Netherlands). The TG reaction in whole blood is carried out in a microfluidic chip (figure 1B) moulded from methyl methacrylate-acrylonitrile-butadiene-styrene (MABS) (chips are injection moulded by Mekora, Aachen Germany). The chip consists of two separately moulded pieces of MABS with a porous matrix disc of 5 mm in diameter and 160 µm thickness (589/1; Whatman, Germany) in their centre. Before assembly the part that bears the microfluidic

channels is treated with a plasma-cleaner (PDC-32G-2, Harrick Plasma, NY, USA) for 15 minutes.

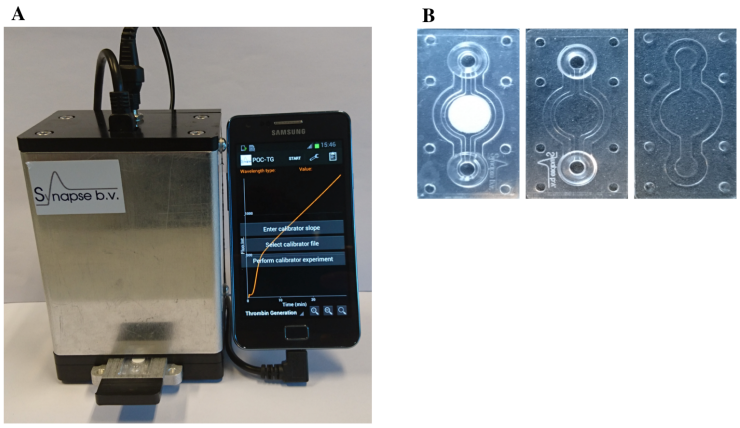

Figure 1. Presentation of the near patient TG system.
(A) The image of the prototype device and connected smartphone with dedicated software. **(B)** Image of an assembled (left) and disassembled (right) two-layer transparent microfluidic (MABS) chip with a porous matrix disc of 5 mm in diameter and 160 µm thickness.

Near Patient TG Assay

Citrated blood (15 µL) was mixed with 15 µL activator solution containing $(ZGGR)_2$-Rhodamine 110 (P_2Rho), recombinant human tissue factor (TF; Innovin®, Dade Behring, Germany) and $CaCl_2$ in 20 mM HEPES, 140 mM NaCl and 5 mg/mL bovine serum albumin (Sigma, the Netherlands) with a pH of 7.35. The P_2Rho was a kind gift of Diagnostica Stago, (France). The final concentrations are 50% volume blood, 1 pM TF, 16.7 mM $CaCl_2$, and 300 µM P_2Rho. To calibrate the fluorescence signal, a second measurement was performed in which 5 µL P_2Rho and 10 µL α2macroglobulin-thrombin (α2M-T) complex (prepared as previously described[16]) were added to the blood. The final thrombin activity at 100 nM was used for calibration of the experiments. The fluorescence signal was recorded with γ_{ex} = 485 nm and γ_{em} = 538 nm. The assay was performed at 37°C.

Models for Thrombogram Analysis

The traditional CAT algorithm describes the thrombin generating capacity of a person from a thrombogram, using the parameters thrombin peak height (peak, nM), area under the curve (ETP, endogenous thrombin potential, nM.min), time to reach 5 nM of thrombin (lag-time, min), and time-to-peak (ttpeak, min)[4]. The time-consuming nature makes the CAT method unsuitable for fast near patient testing. We assumed that the sigmoidal part of the fluorescence curve, until its inflection point, provides relevant information regarding the thrombin generating capacity of

a person. The sigmoidal part of the curve was fitted with a flexible, extended Chapman-Richards growth (CRG) equation, $F=a(1-e^{-bt})c+d(1-e^{-ft})$[17], where F is the fluorescence intensity at time t and a, b, c, d, f, and g are the parameters that determine the shape of the curve. Subsequently, the peakETP (area under the curve until the peak is reached), peak, lag-time, and time-to-peak (ttpeak) were calculated from the first derivative of the simulated curve.

PPP CAT

PPP CAT was performed as previously described[4]. Briefly, the CAT assay was measured in a pre-heated plate fluorometer (Ascent reader, Thermolabsystems OY, Helsinki, Finland). To each well, 80 µl of plasma was added in combination with the TF trigger (final concentration 1 pM) and phospholipid vesicles (20 mol% phosphatidylserine, 60 mol% phosphatidylcholine and 20 mol% phosphatidyl-ethanolamine from Avanti) at a final concentration of 4 µM in Hepes-buffered saline.

Statistics

Statistical analysis was performed with SPSS version 21 (IBM, Inc). Results are expressed as mean and standard deviation (SD). Data were checked for normality and the Spearman or Pearson correlation was calculated accordingly, to verify the association between different parameters. In addition, patients were divided into two groups based on the median of each of the TG parameters, clinical parameters or median drainage volume. Resulting groups were compared for drainage volume and other laboratory/clinical parameters using the Mann-Whitney U test for independent samples for scale variables and the Fisher's exact test for nominal variables.

Results

Comparison of calculation methods

To validate the CRG model-based data analysis procedure, we compared the thrombogram parameters calculated with the CRG model to those obtained with the CAT-based calculation method[18,19]. Citrated blood from one subject was activated with TF (5 pM) and $CaCl_2$ (16.7 mM) in the presence of 300 µM P_2Rho. The increase in fluorescence intensity was recorded over time using the CAT. The CAT-based analysis is displayed in figure 2A. The calibrator data represent the cleavage of substrate by a known amount of α2M-T (solid line). The fluorescence intensity from the experimental mixture (dotted line) was converted in a thrombin concentration using the calibrator. In figure 2B the first derivative of the calibrated curve shows the generation of free thrombin in molar concentration (solid line), after correction for the formation of α2M-T complex (dotted line), that still cleaves

the substrate but does not represent free thrombin activity. This method covers all coagulation stages, including initiation, propagation and termination of TG.

Secondly, the same set of raw data, but only until the inflection point of the sigmoidal curve was fitted using the Chapman-Richard function[17]. In figure 2C, the calibrator data (solid line), the raw data points (dotted line), and the results of the fit (solid red line) are displayed. In figure 2D the goodness of the fit is determined by the difference between the experimental and fitted data. The sum of these differences constitutes the total error of the fit. In figure 2E the first derivative of

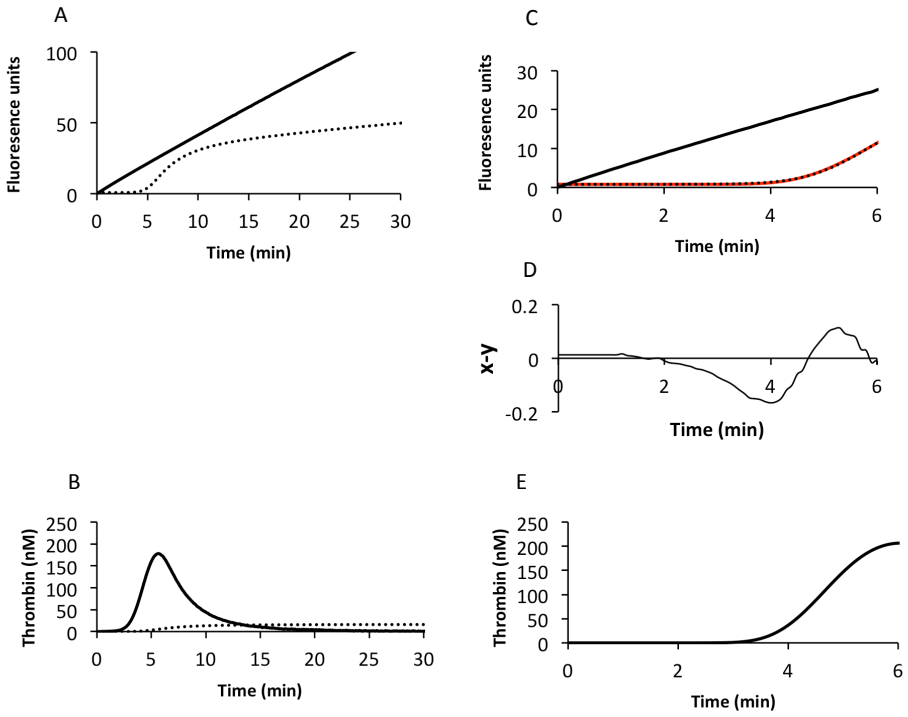

Figure 2. TG curves determined using the CAT method (A,B) and the CRG method (C-E). Citrated whole blood from a healthy donor was triggered with 5 pM TF, 16.7 mM $CaCl_2$ in the presence of 300 µM P_2Rho. The fluorescence intensity was recorded in triplicate measurements. **(A)** The raw calibrator data (solid line) and raw TG data (dashed line) are shown. (**B**) The first derivative of the corrected and calibrated curve (solid line) after correction for the α2M-T complex formation (dashed line) is shown. (**C**) The raw calibrator data (solid line), the raw experimental TG data (dashed line) are shown up to the inflection point, together with the fitted data using the CRG model (red solid line). (**D**) The residues,

$(F_{exp}-F_{fit})^2$ resemble the goodness of the fit. (**E**) The first derivative of the CRG simulated curve until the inflection point is shown.

Thrombogram parameters were calculated for both the CAT and CRG method, and this approach was repeated for 4 additional healthy individuals. To compare the thrombogram parameters obtained with both models, correlation coefficients between each of the TG parameters of the two calculation methods were calculated and were 0.868, 0.998, 0.993 and 0.981 for the (peak)ETP, peak, lag-time and ttpeak, respectively.

The CRG model only considers the data until the peak is reached. Therefore, the peakETP calculated with the CRG model provides limited information about the termination phase of TG, resulting in a relatively lesser correlation with the ETP parameter of the CAT model.

Inter-assay precision

The assay was tested for its accuracy in measuring the calibration factor for which the rate of increase of fluorescence intensity (FU/min) can be converted in molar amounts of thrombin. Citrated blood from one subject was repeatedly (n=10) mixed with α2M-T (100 nM) in the presence of 300 µM P_2Rho. Under the experimental conditions used, we found that during the assay time (10 min), the fluorescence intensity increased linear in time during the full course of the experiment. The coefficient of variation between the 10 calibrator measurements proved to be 13.8%.

Similarly, re-calcified citrated blood from the same healthy subject was repeatedly (n=10) assayed for its TF-induced (1 pM TF) thrombin generating capacity. The resulting thrombogram parameters, displayed as mean ± SD (CV) are shown in table 1. The inter-assay variation of the 5 healthy individuals that were measured to determine the inter-individual variation, as well as that of the 33 patients included for the clinical evaluation, was < 11% for all of the TG parameters. These results are comparable with previously published inter-assay precision in the whole blood CAT[6]. Intra-assay precision could not be assessed, as we are restricted to measuring one sample at a time. Moreover, inter-assay precision was not determined over a time period longer than 1 day due to the storage limitations of whole blood.

Inter-individual variation

The thrombin generating capacity of TF-activated re-calcified citrated blood from 5 healthy subjects was measured in triplicate. The mean and SD for the different parameters between the 5 subjects are displayed in table 1. The inter-individual

variation of the TG parameters obtained with our new device are comparable to those of the whole blood CAT[6].

Table 1. Inter-individual and inter-assay variation in TG parameters.
For the inter-individual variation 5 healthy subjects were tested using the near patient TG device; for the inter-assay variation 1 healthy subject was tested 10 times. TG parameters were determined using the CRG model

	Inter-individual variation N = 5		Inter-assay variation N = 10	
Parameter	Mean±SD	%CV	Mean±SD	%CV
peakETP (nM.min)	154.7±34.3	22	133.5 ± 19	14
Peak (nM)	116.5±26.2	22	102.5 ± 11.9	12
Lag-time (min)	3.18±0.4	13	2.6 ± 0.2	8
ttpeak (min)	5.8±0.4	7	5.1 ± 0.4	8

Near patient whole blood TG in patients undergoing cardiothoracic surgery

To test the applicability of our new method, we studied 33 patients scheduled for CABG with the use of a heart lung machine for extracorporeal circulation. To compare the results obtained with our new method to the golden standard in TG, measurements were also performed in PPP. Patients were divided in two groups based on the median of drainage volume, resulting in a group of 'severe' and 'mild' bleeders. General patient characteristics, as well as pre- and post-operative variables of all groups are shown in table 2 in the supplemental information. A statistically significant difference was found between the two groups for the peakETP and the peak of the near patient data and for the ETP of the PPP data. The resulting parameters displayed as mean ± SD per group with their respective P-value are for the peakETP 162.8 ± 38.1 versus 120.3 ± 35.9 (P = 0.002), the peak 136.7 ± 40.4 versus 106.0 ± 29.6 (P = 0.011) and the ETP (PPP) 1244.5 ± 274.1 versus 1025.1 ± 216.4 (P = 0.017) for the severe versus mild bleeders, respectively. Other parameters that differed significantly between the two groups include the patient's weight (87.9 ± 10.9 versus 76.1 ± 13.9; P = 0.015), their BMI (29.1 ± 2.3 versus 25.1 ± 3.9; P = 0.001), and the administered volume of colloids during the first 24h on the intensive care unit (21.4 ± 10.8 versus 9.2 ± 6.4; P < 0.001).

Alternatively, to determine the predictive potential of different parameters for bleeding, patients were divided in two groups based on the median of the general characteristics, the peri- and post-operative variables, and the clinical characteristics. Next, the difference in drainage volume, was compared between these two groups (table 3 in the supplemental information). A statistically significant difference in drainage volume was found when patients were divided based on the median BMI, post-operative colloid administration, the peakETP, the

peak parameters of the near patient data, and the pre-operative fibrinogen concentration. Individual data of the drainage volume for the two groups stratified according to the median of the peakETP and BMI are shown in figure 3A, and figure 3B, respectively. Interestingly, the combination of peakETP, lag-time and BMI further enhanced the prediction of the drainage volume (figure 3C).

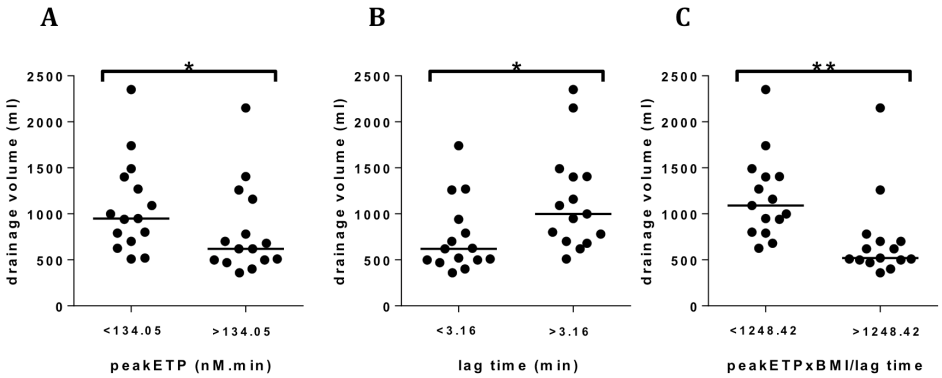

Figure 3. Drainage volume in patients undergoing cardiothoracic surgery stratified according the median peakETP (A) as assessed by our near patient device, the median BMI (B), and a combined parameter based on the peakETP, lag-time and BMI (C).
33 patients undergoing cardiothoracic surgery were divided into two groups based on either the median peak-ETP (**A**), BMI (**B**), or a combined parameter based on the peakETP, lag-time and BMI. Individual values of drainage volume are shown, together with the mean ± SEM. * $p<0.05$, ** $p<0.005$, and *** $p<0.001$ (Mann-Whitney U test)

Discussion

The present study demonstrates the technical development and clinical testing of a whole blood TG method as a near patient test. Our new whole blood device is characterized by a good technical performance, comparable to the standard CAT method. The high turnover time (up to 60 min) is still a major bottleneck for the current TG[20]. Not only have we been able to reduce the size of the device, with our CRG calculation model we decreased the turnover time of our new test to less than 20 min. To achieve the short measurement time, an altered calculation method is needed, only considering the data until the peak of the TG curve.

We tested the clinical applicability of the device in a population of patients N = 33 undergoing elective CABG. These patients are known to have a high bleeding risk due to the invasive nature of the surgery[21,22]. The extracorporeal circulation is believed to be one of the main contributors to the bleeding risk. The circuit causes major haemostatic disturbances e.g. due the dilution of coagulation factors as a result of the priming fluid of the machine[23,24]. Currently there are no tests available

that accurately predict the risk of blood loss of a patient planned to undergo surgery. The coagulation tests that are performed pre-operatively are mainly used to identify coagulation disorders that increase the risk of peri-operative bleeding[25]. However, these tests lack the sensitivity to pick up subtle inter-individual differences between patients that do not have any obvious coagulopathy. The more recently emerged viscoelastic tests for near patient testing, including thromboelastography and rotational thromboelastometry, are primarily used to aid in the selection of the optimal allogeneic blood products or coagulation factor concentrates during/after clinical bleeding[20]. However, these viscoelastic tests have little predictive value in terms of peri-operative blood loss[26,27].

TG measurements in plasma proved to be indicative for blood loss in patients undergoing CABG[28,29]. Despite the relatively small number of patients included, our study confirms these results, as patients with a volume below the median drainage volume, were characterized by a significantly lower ETP in plasma. Dividing patients based on the median of the drainage volume, also resulted in significant differences in peakETP and peak determined by the near patient whole blood TG device, as well as the BMI. This is in accordance with other studies that have shown that patients with a lower BMI are at a higher risk regardless of the type of cardiac surgery with CPB[30,31].

To determine the predictive capacity of our near patient measurements, we divided patients based on the median of pre- and postoperative determined parameters, and compared the drainage volume between both groups. Interestingly, patients with a peakETP below the median, experienced significantly more drainage volume compared to those with a peakETP above the median. Moreover, a division based on the peak data of our new device resulted in a similar significant difference between the two groups.

Besides our near patient TG parameters, the BMI was a predictive indicator for the amount of drainage volume when subjected to this form of analysis. Interestingly, the combination of the peakETP, the lag-time, and the BMI of the patient, proved to be a better predictor of the drainage volume than each parameter separately. In accordance with previous results, the pre-operative fibrinogen concentration shows a similar trend[32].

We do acknowledge the limitation of the study size and understand that, despite the statistical significance, this study should be extended to larger patient populations. Additionally, further clinical studies are necessary to determine its importance in the monitoring and therapeutic guidance of other bleeding and thrombotic pathologies. A possible limitation of our device is that, due to the external mixing of reagents, a technician and laboratory environment are still

necessary. We are currently busy with the implementation of the reagents in the chips. Furthermore, we are optimizing a quality control that can be run daily to verify proper functioning of the device. Before implementation of the device in clinical settings, the effect of common interferents such as bilirubin, lipid particles and commonly co-administered drugs should be further investigated. Of note, the haemoglobin and creatinine levels pre-surgery did not correlate with the pre-surgery TG parameters (data not shown). Indirect effects of co-administered drugs and other surgery-specific effects should be assessed prior to clinical use of our device. In this study, the patient group was too small to draw any conclusions regarding these effects. Of course, anticoagulant drugs will have an influence on the outcome of our test as they influence the coagulation system of the patient. Usually, patients are asked to discontinue this medication before surgery, but any remaining of these medications will influence TG and hence blood loss.

Importantly, despite the small sample size and the described limitations, our data suggest an added clinical value for our TG device being indicative of the bleeding risk after cardiac surgery. A test, such as the near patient TG, that can be performed in the vicinity of the patient and hence gives a fast result, has the added benefit that treatment can be initiated faster. This in turn makes the treatment more effective as time is of utmost importance in a clinical setting[33]. Ultimately, after further validation and simplification, our device may be suitable for use outside a laboratory/hospital environment e.g. in the general practitioner's office. This will reduce the number of routine hospital visits and therewith improve the quality of life of patients.

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Supplementary information

Table 2. Distribution of patient characteristics, peri- and post-operative variables. Patients are divided into two groups based on the median of the drainage volume. Group 1 had a drainage volume of < 780 ml group 2 had a drainage volume > 780 ml.

Variable	All patients N=33		Group 1 N=17		Group 2 N=16		P-value Mann-Whitney U-test / Fisher's exact test
	Mean	SD	Mean	SD	Mean	SD	
General characteristics							
Age (years)	66.8	8.7	67.1	10.0	66.5	7.5	0.958
Gender (% female)	24%		35%		13%		0.225
Length (cm)	173.6	7.8	173.5	7.6	173.8	8.5	1.000
Weight (kg)	82.2	13.6	87.9	10.9	76.1	13.9	0.015
BMI	27.2	3.7	29.1	2.3	25.1	3.9	0.001
Intraoperatively							
Heparin-total (IU/kg)	94.6	59.1	99.2	75.3	89.8	36.8	0.510
Protamin-total (mg/kg)	3.6	0.5	3.4	0.4	3.8	0.6	0.087
Tranexamic acid (mg/kg)	36.8	13.2	32.7	6.0	41.2	17.1	0.063
Crystalloids (ml/kg)	14.7	9.8	11.6	7.2	17.3	11.5	0.168
Colloids (ml/kg)	0.7	1.7	0.7	1.5	0.7	1.9	1.000
Total packed cells (ml/kg)	0.6	2.1	0.2	0.9	1.1	2.9	0.736
FFP (ml/kg)	0.0	0.0	0.0	0.0	0.0	0.0	NA
Platelet concentrates (ml/kg)	0.3	1.3	0.0	0.0	0.7	1.9	0.557
Fibrinogen (mg/kg)	1.3	5.4	0.0	0.0	2.6	7.7	0.557
Fluid in Cell Saver (ml)	439.2	230.2	474.9	240.0	401.3	220.8	0.345
Fluid in Ultrafiltration (ml)	333.3	578.1	317.7	604.4	350.0	568.0	0.873
Time on bypass (min)	82.0	17.3	81.1	17.6	83.1	17.5	0.557
Time X-clamping (min)	59.3	16.1	59.2	17.1	59.4	15.6	0.790
Duration surgery (min)	205.0	45.0	205.5	49.8	204.6	40.9	0.763
Number of CABG	3.6	1.1	3.4	1.3	3.9	0.8	0.465
Postoperatively							
Crystalloids (ml/kg)	27.8	10.0	26.5	9.1	29.3	11.0	0.276
Colloids (ml/kg)	15.1	10.7	9.2	6.4	21.4	10.8	0.000
Total packed cells (ml/kg)	3.6	7.3	0.4	1.1	6.9	9.5	0.053
FFP (ml/kg)	1.0	3.5	0.0	0.0	2.0	4.9	0.363
Platelet concentrates (ml/kg)	1.0	2.2	0.0	0.0	2.0	2.8	0.068
Fibrinogen (mg/kg)	3.2	10.2	0.0	0.0	6.5	14.1	0.363
Re-thoracotomy (% of patients)	9%		0%		18.8%		0.103
Laboratory parameters before surgery							

peakETP (nM.min)	142.2	42.4	162.8	38.1	120.3	35.9	0.002
peak (nM)	121.8	38.4	136.7	40.4	106.0	29.6	0.011
lag-time (min)	3.2	0.5	3.2	0.4	3.3	0.5	0.309
ttpeak (min)	5.3	0.7	5.3	0.7	5.3	0.7	0.958
PPP ETP (nM.min)	1138.1	268.2	1244.5	274.1	1025.1	216.4	0.017
PPP peak (nM)	267.4	75.6	286.6	78.5	246.9	69.0	0.094
PPP lag-time (min)	4.2	1.9	4.4	2.0	4.0	1.9	0.231
PPP ttpeak (min)	6.4	2.3	6.6	2.5	6.2	2.2	0.363
Fibrinogen concentration (g/L)	3.6	0.8	3.9	0.9	3.4	0.6	0.127
Blood loss							
Blood loss during surgery (ml)	1514.6	699.0	1529.4	586.5	1499.0	821.5	0.465
Drainage volume (ml)	928.2	485.3	585.0	123.8	1292.8	459.1	0.000
Total blood loss (ml)	2442.8	894.2	2114.4	517.1	2791.5	1080.9	0.031

BMI, Body Mass Index; CABG, Coronary Artery Bypass Grafting; ETP, Endogenous Thrombin Potential; ttpeak, time-to-peak; FFP, Fresh Frozen Plasma; SD, standard deviation.

Table 3. Distribution of the drainage volume. Patients are divided into two groups based on the median of the indicated parameter.

Variable	< Median N=17		> Median N=16		P-value Mann-Whitney U-test
	Mean	SD	Mean	SD	
General characteristics					
Age (years)	944.7	580.2	910.6	377.7	0.683
Length (cm)	1025.0	598.6	825.3	313.6	0.557
Weight (kg)	1030.9	521.5	819.1	433.3	0.157
BMI	1103.2	484.3	742.19	424.9	0.005
Intraoperatively					
Heparin-total (IU/kg)	825.6	407.7	1037.2	548.2	0.276
Protamin-total (mg/kg)	943.4	481.3	913.8	503.4	0.929
Transexamin acid (mg/kg)	771.5	275.5	1094.7	603.4	0.136
Crystalloids (ml/kg)	933.2	514.0	922.8	569.7	0.736
Fluid in Cell Saver (ml)	928.2	302.9	928.1	635.1	0.231
Time on bypass (min)	814.7	344.9	1048.8	587.7	0.292
Duration X-clamping (min)	898.8	470.9	959.9	513.7	0.736
Duration surgery (min)	887.1	385.1	971.8	583.3	0.958
Number of CABG	778.8	302.9	1086.9	593.6	0.118
Postoperatively					
Crystalloids (ml/kg)	862.4	464.8	998.1	511.7	0.382
Colloids (ml/kg)	738.2	313.1	1130.0	559.9	0.019

Laboratory parameters before surgery					
peakETP (nM.min)	1086.5	480.3	760.0	444.7	0.006
peak (nM)	1049.7	476.3	799.1	475.3	0.031
lag-time (min)	856.2	536.3	1004.7	428.3	0.094
ttpeak (min)	897.9	539.8	960.3	435.3	0.309
PPP ETP (nM.min)	1070.3	558.3	777.2	350.3	0.118
PPP peak (nM)	1024.1	573.6	826.3	360.5	0.382
PPP lag-time (min)	932.9	408.9	923.1	569.2	0.510
PPP ttpeak (min)	968.2	388.7	885.6	580.9	0.146
Fibrinogen concentration (g/L)	1114.4	565.8	730.3	282.6	0.034
Blood loss					
Blood loss during surgery (ml)	914.7	366.7	912.0	612.0	0.136
Total blood loss (ml)	726.2	236.7	1142.8	589.3	0.041

BMI, Body Mass Index; CABG, Coronary Artery Bypass Grafting; ETP, Endogenous Thrombin Potential; ttpeak, time-to-peak.

Chapter 3

Thrombin Generation in Zebrafish Blood

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Abstract

Background To better understand hypercoagulability as an underlying cause for thrombosis, the leading cause of death in the Western world, new assays to study ex vivo coagulation are essential. The zebrafish is generally accepted as a good model for human haemostasis and thrombosis, as the haemostatic system proved to be similar to that in man. Their small size however, has been a hurdle for more widespread use in haemostasis related research.

Methods In this study we developed a method that enables the measurement of thrombin generation in a single drop of non-anticoagulated zebrafish blood. Scanning electron microscopy for the visualization of the fibrin network.

Results Pre-treatment of the fish with inhibitors of FXa and thrombin, resulted in a dose dependent diminishing of thrombin generation, demonstrating the validity of the assay. In order to establish the relationship between whole blood thrombin generation and fibrin formation, we visualized the resulting fibrin network by scanning electron microscopy.

Conclusion Taken together, in this study we developed a fast and reliable method to measure thrombin generation in whole blood collected from a single zebrafish. Given the similarities between coagulation pathways of zebrafish and mammalians, zebrafish may be an ideal animal model to determine the effect of novel therapeutics on thrombin generation. Additionally, because of the ease with which gene functions can be silenced, zebrafish may serve as a model organism for mechanistical research in thrombosis and haemostasis.

Introduction

Thrombosis remains a leading cause of death in the western world. Aside from mortality, significant morbidity occurs from thrombotic events. The causes of this hypercoagulability are becoming more and more clear with an enhanced knowledge of haemostasis and the development of new coagulation assays. Most of this knowledge results from extensive in vitro biochemical characterization of blood coagulation, whereas studies investigating blood coagulation in vivo are limited.

Due to the availability of knockout technology, genetic studies of thrombosis in mice are popular. Nonetheless, the time consuming and labour-intensive process of generating knockouts restricts these studies. The zebrafish is generally accepted as a good model for mammalian haemostasis and thrombosis due to the presence of coagulation factors, platelet receptors and its response to anticoagulant drugs commonly used in clinical treatment[1]. In addition, haemostatic pathways in zebrafish proved to be similar to those in man[2–4]. Interestingly, the use of zebrafish enables large scale mutagenesis screening to identify novel genes involved in haemostasis and thrombosis[5,6].

The small size of zebrafish has been a hurdle in thrombosis and haemostasis research since most of the conventional coagulation assays require large amounts of plasma. Jagadeeswaran *et al.* optimized a total coagulation activity screening assay using small quantities of zebrafish plasma, by adding human fibrinogen and measuring fibrin formation by turbidimetry to probe thrombin formation[7]. Additionally, they developed an ultra-sensitive kinetic method to identify specific pathway defects in small quantities of plasma. Disadvantages of such assays include the requirement of multiple zebrafish and the use of plasma, not considering the effect of thrombocytes and erythrocytes on coagulation.

At present, mainly end-point assays are used to detect coagulation defects. These assays simply measure the time it takes for a platelet-poor plasma sample to clot, i.e. when the first traces of fibrin are formed. However, fibrin formation already starts in the presence of tiny amounts of thrombin (≈1 nM)[8]. Thus, the vast majority of thrombin takes place after fibrin formation[9], suggesting that clotting time-based assays only measure the initiation and not the propagation phase of coagulation. Importantly, correct functioning of the haemostatic system proved to be dependent on the total amount of thrombin that is formed during coagulation[10]. We recently developed a reliable method to measure thrombin generation in a drop of whole blood, thereby bringing coagulation one step closer to physiology[11]. In this study, we further optimized this method enabling the determination of thrombin generation in a drop of non-anticoagulated whole

blood obtained from a single zebrafish. Thrombin generation proved to be sensitive to pre-treatment of the fish with inhibitors of FXa and thrombin. Furthermore, we visualized the resulting fibrin network by scanning electron microscopy (SEM) in order to analyse the density and dimensions of the fibrin strands.

Materials and methods

Reagents

20 mM Hepes buffer (pH 7.35) containing 5 mg/ml bovine serum albumin (BSA) and 140 mM NaCl (BSA5) or 60 mg/ml bovine serum albumin (BSA60) were prepared as described previously[12]. The rhodamine-based substrate P_2Rho was a kind gift of Diagnostica Stago. The calibrator, $\alpha_2$macroglobulin-thrombin (α_2M-T) complex was prepared in-house as described previously[12]. Rivaroxaban (Xarelto) was from Bayer and melagatran was a gift from AstraZeneca.

Blood collection and treatment of zebrafish

This study was carried out in strict accordance with the recommendations in the guide for the use of laboratory animals of the university of Liège. The protocol was approved by the committee on the ethics of animal experiments of the university of Liege Permit number LA 1610002. Blood was collected from adult (male and female) wild type zebrafish (*Danio rerio)* as described previously[7]. Briefly, with a small pair of scissors, an incision was made at the lateral side of the fish just posterior of the dorsal fin, thereby transecting the dorsal vein/artery. From the blood welling up in the wound, 5 µl was collected for further analysis.

For anticoagulant treatment, fish were sedated with a high dose of tricaine solution (1.6 mg/ml). Fish were dried with paper and weighed. Only fish weighing less than 1 gram were used and injected intraperitoneally with 20 µl/g of the indicated anticoagulant in phosphate buffered saline (PBS) and 0.25% phenol red (to monitor the injection process). After injection, fish were allowed to recover for 30 minutes after which blood was collected as described above.

Thrombin generation measurement

For thrombin generation, an adapted protocol was developed based on our whole blood thrombin generation assay[11]. Collected whole blood (5 µl) was mixed with 5 µl of HEPES buffer containing the P_2Rho substrate (final concentration (fc) 300 µM). 5 µl of this mixture was put on a paper disk and covered with mineral oil to prevent evaporation. The lag-time phase of the thrombin generation experiment was started as soon as the incision for the blood withdrawal was made. Calibration was done by adding 5 µl of whole blood to 5 µl of HEPES buffer containing P_2Rho (fc 300 µM), α2M-thrombin calibrator (fc 100 nM) and citrate (fc 9,8 mM).

Fluorescence was recorded with a fluorescence detector (ESElog, Qiagen) with λ_{ex} = 485 nm and λ_{em} = 538 nm. All experiments were performed at 37°C.
Analysis of the fluorescence tracings to yield the thrombogram and corresponding parameters was performed with a modified method, considering only the thrombin generation until the peak is reached. From the resulting thrombogram the following parameters were calculated: lagtime (min), peak (nM, maximal thrombin concentration), peak-endogenous thrombin potential (ETP, nM.min, area under the thrombin curve until the peak is reached), time to peak and velocity (nM/min, maximal rate of thrombin generation).

The human samples were analysed using the plasma CAT as previously described[12] in the presence or absence of either rivaroxaban or melagatran.

Plasma samples for normal pool plasma

After approval of the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Centre) 24 healthy adult volunteers who did not take any drugs for at least two weeks gave full informed written consent according to the Helsinki declaration. Blood was collected aseptically by antecubital puncture into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System).

For the normal pooled plasma (NPP), blood from the 24 volunteers was prepared by centrifuging the blood at 2900g during 10 min at room temperature. Plasma was aspirated and the procedure was repeated. Plasmas were pooled and further ultra-centrifugation (100000g, 70 min) was carried out. Aliquots of 1 ml were stored at -80°C until use.

SEM analysis

After thrombin generation was determined in whole blood, the clots were prepared for visualization by SEM. The clots were fixated by adding 2.5% glutaraldehyde (grade I, Sigma Aldrich, St. Louis, Missouri) in PBS (Sorensen's, pH 7.2) (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour at room temperature and then placing it at 4°C overnight. The following day, the glutaraldehyde solution was removed and the samples were repeatedly (5x) washed with PBS. As a secondary fixation, the samples were placed in osmiumtetroxide (OsO_4, 1%) diluted in sodium cacodylate (200 mM, pH 7.4) (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour at room temperature. Consecutively, the clots were dehydrated in ethanol (30%, 50%, 70%, 90% and 3 times at 100%) for 3 minutes. The samples were then treated with a hexamethyldisilane/ethanol solution for 3 minutes and in hexamethyldisilane (Sigma Aldrich, St. Louis, MO, USA) for 10 minutes. The samples were removed

from the wells, left to dry and coated with gold. Analysis was performed on a desktop SEM (Phenom-World, Eindhoven, the Netherlands).

Statistical analysis

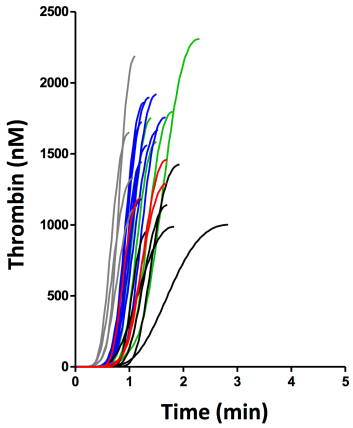
For statistical analysis of the data GraphPad Prism Software was used. Differences between two groups were evaluated by the one-way ANOVA test or the Mann Whitney U test.

Results

Development of an assay to measure thrombin generation in zebrafish

In a first set of experiments, thrombin generation was determined in 5 µl whole blood collected from single zebrafish, using a method comparable to whole blood calibrated automated thrombography[11]. The limited blood volume of a single zebrafish, impedes the performance of both a thrombin generation and calibrator measurement. Since calibrator measurements performed on the blood of 28 different fish in 5 independent experiments demonstrated an acceptable variation (mean CV of 14%), the average calibrator slope per experiment was used for thrombin generation calculations.

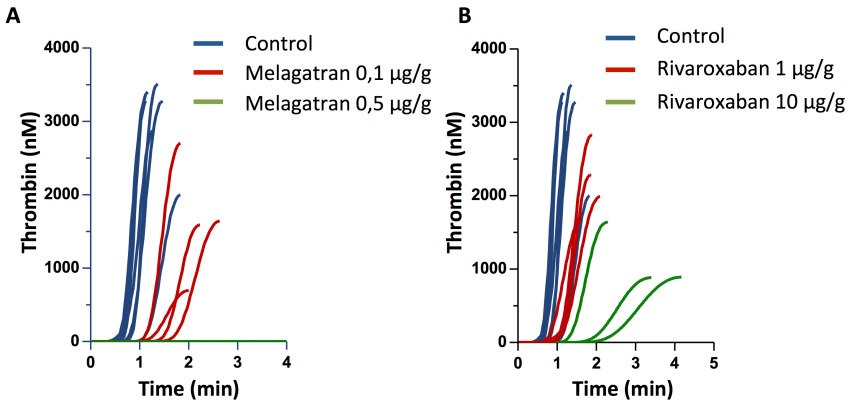
Subsequently, thrombin generation was measured in 5 independent experiments, each consisting of 3 to 5 thrombin generation measurements. Thrombin generation curves, together with the average and %CV of the individual thrombin generation parameters are shown in figure 1. The thrombin enzyme from zebrafish clearly proved to be capable of cleaving our rhodamine-based substrate. Interestingly, thrombin generation measured in individual fish showed a similar amount of inter-individual variation as in humans[11]. Striking differences with human thrombin generation include the much shorter lag-time, and the increased peak height and velocity index. Taken together, our results demonstrate the feasibility of measuring thrombin generation in whole blood collected from a single zebrafish.



	Mean	Mean CV (%)
Peak ETP (nM.min)	707.46	33.78
Peak (nM)	1504.00	17.75
Lag time (min)	0.50	13.05
Time to peak (min)	1.53	17.51
Velocity (nM/min)	3197.60	26.14

Figure 1. Thrombin generation curves and parameters in zebrafish.
Thrombin generation was measured in 5 independent experiments, in a total of 25 zebrafish. **(A)** Thrombin generation curves are shown. Each colour represents an independent experiment. **(B)** Mean thrombin generation parameters and their CV are calculated.

In a next set of experiments, the effect of pre-treatment of the zebrafish with thrombin or FXa inhibitors on thrombin generation parameters was tested. Zebrafish were treated with the indicated compounds and doses for 30 minutes as mentioned before, followed by blood collection and thrombin generation measurements. Thrombin generation curves of the individual zebrafish are shown in figure 2. Results are expressed as the percentage inhibition compared to a control group of vehicle-treated fish (n = 8) and are representative for 2 independent experiments.



C

	Melagatran (0.1 µg/g ; n = 4)		Rivaroxaban (1 µg/g ; n = 4)		Rivaroxaban (10 µg/g ; n = 3)	
	% inhibition	P-value*	% inhibition	P-value$	% inhibition	P-value$
Peak ETP (nM.min)	27.28	ns	-8.18	ns	11.13	ns
Peak (nM)	46.58	**0.01**	29.25	ns	63.36	**< 0.01**
Lag time (min)	-102.03	**0.01**	-18.09	ns	-150.41	**< 0.05**
Time to peak (min)	-61.40	**0.01**	-39.13	ns	-143.23	**< 0.01**
Velocity (nM/min)	64.25	**0.01**	56.93	ns	84.97	**< 0.01**

Figure 2. Inhibition of thrombin generation in zebrafish by thrombin or FXa inhibition. Zebrafish were treated with Melagatran **(A)** or rivaroxaban **(B)** at the indicated doses for 30 min, followed by thrombin generation measurements. **(C)** Results are expressed as the percentage inhibition compared to a control group of vehicle-treated fish (n = 4). Results are representative for two independent experiments. Statistical differences between treatment and control groups were performed with *Mann-Whitney U-test or $1-way ANOVA. (ns = not significant)

As to the thrombin inhibition, treatment with melagatran concentrations above 0.5 µg/g completely blocked thrombin generation, clearly illustrating that the observed fluorescent signal is thrombin- related. A concentration of 0.1 µg/g melagatran significantly inhibited thrombin generation, as evident from the significantly decreased peak height and reduced lag-time, time to peak and velocity index. The dosage needed to inhibit thrombin generation in zebrafish was considerably lower than concentrations used in in vitro experiments with normal pooled plasma. In these experiments a concentration range of 1073 to 3575 µg/g was needed to get a dose dependent inhibition of thrombin generation. Comparable results were obtained upon injection of another thrombin inhibitor, hirudin (4 µg/g) (data not shown). Additionally, inhibition of FXa by rivaroxaban

treatment (concentrations of 1 and 10µg/g) resulted in a similar inhibition of the thrombin generation. The concentration was in contrast with the in vitro results in normal pooled plasma, in which lower concentrations from 72,65 µg/g rivaroxaban already resulted in inhibition of thrombin generation. However, the dosages used in the zebrafish were much closer to the prescribed dose for adults of 20 mg/day (± 0.29 µg/g)

SEM analysis of fibrin clots

Given the accelerated and increased thrombin generation in zebrafish compared to humans, we decided to analyse the fibrin ultrastructure at the end of a thrombin generation experiment by SEM. The mineral oil that was used to prevent evaporation was removed and the fibrin that was formed was fixated for visualization with SEM. A representative image of the fibrin network is shown in figure 3. Analysis of the clot revealed a much denser network compared to humans, composed of thin fibrin fibres. Interestingly, the structure of the network is comparable to a human fibrin network triggered with high tissue factor concentrations (data not shown). The entrapped red blood cells and thrombocytes in the fibrin network, might suggest their participation in the process of coagulation.

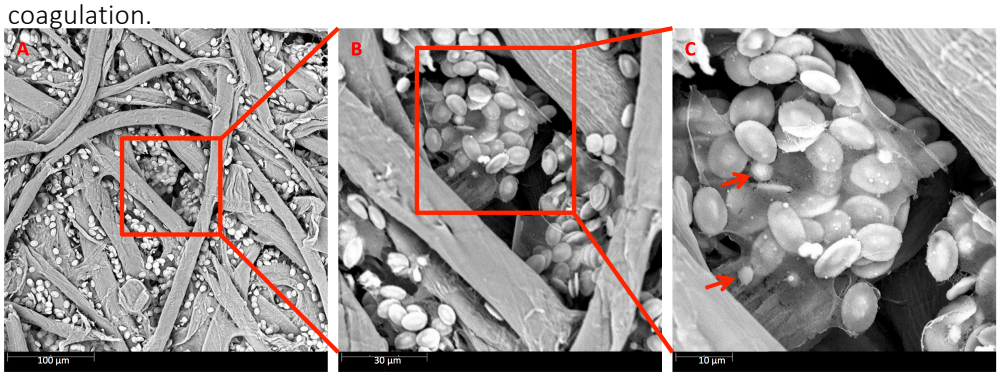


Figure 3. Scanning electron microscopy (SEM) of zebrafish blood clots. (A) Representative image of SEM analysis of blood clots formed during thrombin generation measurements. **(B-C)** Sequential enlargements of the fibrin network with entrapped red blood cells. Arrows in **(C)** indicate platelets.

Discussion

In this study, we optimized an assay to measure thrombin generation in whole blood obtained from a single zebrafish. A major advantage compared to previous tests, is the use of whole blood instead of plasma, resulting in a quick and reliable screening tool to measure blood coagulation in zebrafish. Furthermore, as the

measurement starts directly after the blood collection, the use of an anticoagulant that may influence coagulation is avoided.

The suitability of the zebrafish model to study haemostasis depends hugely on the degree of similarity between the zebrafish and mammalian systems. Previous studies have established that, despite the evolutionary distance, the major coagulant and anticoagulant pathways are similar between zebrafish and mammalians, as evident from the presence of a comparable contact activation system, extrinsic pathway and common pathway[3]. The effects of prothrombin deficiency in zebrafish embryos, ranging from early morphological defects and internal bleeding to occasional bleeding in the brain at a later stage, suggest that the mechanism of thrombin signalling is conserved across vertebrates[13]. To our knowledge, our method is the first that allows measurement of thrombin generation in whole blood obtained from a single zebrafish. Thrombin generation experiments in zebrafish showed a similar inter-individual variation as in humans[11]. However, thrombin generation proved to be significantly accelerated and increased compared to humans. With the current blood collection method, using a pair of scissors, exposure of the blood to tissue factor is inevitable. Therefore, the current method is suits well for tests related to the intrinsic coagulation system.

By pre-treating zebrafish with thrombin inhibitors, we clearly provided evidence that the observed fluorescent signals in our assay are the result of thrombin. The treatment with thrombin inhibitors, hirudin and FXa was found to significantly inhibit thrombin generation in zebrafish. Hirudin microinjections into early zebrafish embryos were previously shown to inhibit fibrin forming activity and to cause abnormal development, suggesting a role for thrombin in early development[14]. Furthermore, Jagadeeswaran *et al.* demonstrated that warfarin has similar effects in zebrafish compared to mammals, illustrating that vitamin K-dependent pathways in fish are comparable to human pathways[3,7]. Interestingly, as all tested anticoagulant therapy was found to diminish thrombin generation in zebrafish, zebrafish may be used as an in vivo model system to test the pharmacokinetic and pharmacodynamic aspects of (novel) therapeutics on thrombin generation.

As to the fibrin formation, the appearance of a dense fibrin network composed of thin fibrin fibres is in line with the accelerated and increased thrombin generation. Indeed, high versus low concentrations of thrombin lead to dense networks of thin fibres versus permeable clots composed of thick woven fibrin strands, respectively[8,15,16].

In conclusion, we developed a fast and reliable method to measure thrombin generation in whole blood collected from a single zebrafish. Given the huge

similarities between coagulation pathways of zebrafish and mammalians, zebrafish may be an ideal in vivo model to determine the effect of novel therapeutics on thrombin generation. Additionally, because of the ease with which gene functions can be silenced, zebrafish may serve as a model organism for further mechanistical research in thrombosis and haemostasis.

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Chapter 4

On the Causes of Inter-Laboratory Variability in Calibrated Thrombin Generation Measurement.

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Manuscript in preparation

Abstract

Background The thrombin generation capacity of plasma is an important determinant of the individual thrombotic or bleeding tendency, which makes it a promising clinical variable. At this moment however, its implementation in routine clinical practice is hindered by a relatively high variability between different laboratories. Efforts to improve this variability by normalisation have improved the comparability of some parameters and yet worsened others.

Methods we analysed 34 sets of fluorescence curves of the same sample of reference plasma that were obtained under "real life" conditions in 12 different clinical laboratories throughout France, Belgium and Switzerland.

Results Our analysis revealed two major sources of inter-laboratory variability: Errors due to the calibration procedure and errors due to insufficient temperature control. About 30% of experimental variation can be attributed to the former and about 20% to the latter.

Conclusions Elimination of these errors reduces the total inter-laboratory variability of the area under the thrombin generation curve from 16.3 to 8.1% and that of the peak thrombin activity measured from 17.4 to 8.6%.

Introduction

Thrombin generation, potential benefits of its introduction into clinical practice

Measuring the thrombin level in clotting plasma is an established tool in blood coagulation research since around 1950 but the technique was too laborious for routine clinical use[1,2]. The evolution of the method to the continuous measurement of thrombin by its cleavage of an added fluorogenic substrate increased throughput to around 24 samples per hour[3,4]. The increased throughput triggered a widespread interest in this approach. Throughout the last decades considerable evidence has been published that the amount of thrombin activity that develops in a sample of clotting blood(-plasma) is an indicator of the risk of bleeding[5–8] as well as venous thrombosis[9–17]. It also relates to the risk of arterial thrombosis, be it in a less straightforward manner[18–20]. It is a common denominator of the effect of all anticoagulants[21–28] and, when it is measured in platelet rich plasma, it is inhibited by platelet inhibitors[29,30]. Therefore, it has a promising outlook for use as a clinical parameter.

In calibrated automated thrombography (CAT) the amount of thrombin in the sample in which thrombin is generating (the staple sample), is calculated by comparison to the fluorescence that develops in a separate sample of the same plasma in which a constant known amount of thrombin activity converts the substrate (the calibrator sample). In this way, the value of the calibration factor (CF) can be read at every level of fluorescence. The same plasma needs to be used in both the thrombin generation (TG) and the calibrator experiment because e.g. the colour of individual plasmas influences fluorescence in a different way.

Inter-laboratory variability as an obstacle for clinical implementation

The main issue preventing broad clinical use of TG is the relatively high inter-laboratory variability of its results[31]. This has been attributed to a lack of automation and because of its sensitivity to pre-analytical variables[32]. Rigorous standardization of the pre-analytical and analytical procedure and normalization relative to a reference plasma indeed improved the CV[33,34]. A multicentre study from French-speaking countries (France, Belgium, Switzerland) carried out under “real life” conditions in clinical and research laboratories showed that the use of a common reference plasma could improve inter-laboratory variability of most TG parameters except in cases of frank hypo-coagulability[35]. In the present work, we utilized the original fluorescence traces from the common reference plasma used in this study to investigate possible causes of the observed variability.

It has been observed that TG is “inversely” temperature sensitive: the lower the temperature, the higher the thrombin activity found[36]. Consequently, the TG test is

critically dependent upon the preheating of the samples: insufficient preheating makes the temperature rise during the first minutes of the measurements, leading to an underestimation of the calibrator activity, hence causing an overestimation of the CF, and finally resulting in an underestimation of the thrombin concentration. Moreover, the temperature equilibration process largely coincides with the lag-time in the test sample, and thus is certain to influence this variable. In so far as it extends beyond the lag-time, insufficient temperature control will influence the course of TG as well. The combination of underestimation due to delayed heating of the calibrator and enhancement of TG due to delayed heating of the test sample will result in unpredictable overestimation of the recorded values of Endogenous Thrombin Potential (ETP) and Peak.

The aim of the current study was to evaluate the sources of inter-laboratory variation and to quantify the sources of the variability, notably temperature. Moreover, its aim is to propose the most appropriate strategy for improvement of the assay.

Materials and methods

Data set description

Graphs of fluorescence (in arbitrary units (AU)) against time of both calibrator curves and TG curves were kindly provided by the labs that partook in the study of Perrin[35]. In total we obtained the data of 34 separate experiments in which samples of the same reference plasma were measured in 12 different labs on different days. A detailed description of the experimental conditions and protocol can be found in the original article[35]. In short, a unique and common normal reference plasma (reference Plasma Stago®, Diagnostica Stago Asnières) was used in a standard TG experiment at 5pM tissue factor (TF) in 34 separate experiments performed in 12 laboratories. In the calibration experiments the α2macroglobulin-thrombin complex (α2M-T), Thrombin Calibrator™ (Diagnostica Stago Asnières)) was added as the equivalent of constant thrombin activity. The different participating laboratories used different batches of calibrator. No instruction was given as to preheating of the plates prior to the measurement. The mean values, standard deviations and variation coefficients of thrombin generation parameters for initial data set can be found in table 1.

Table 1. Mean values, standard deviations and variation coefficients of TG parameters for initial data set.

	ETP, nM·min	Peak, nM	ttpeak, min	Lagtime, min
Mean	1553.7	268.0	5.90	2.97
Standard deviation	252.8	46.5	0.48	0.35
Variation coefficient, %	16.3	17.4	8.2	11.7

Temperature control estimation

The stability of the temperature throughout the experiment was assessed from the raw fluorescent data of the calibration curves by means of a specially designed program written by us in Mathematica 10.0 (Wolfram Research). The code of the program is provided in Supplementary materials. Its algorithm is based on the use of the graph of the first derivative of fluorescence (dF/dt) against the fluorescence (F) itself previously defined as the "diagnostic plot"[37,38]. At constant temperature dF/dt is highest at zero time (F_0) because substrate concentration is highest and the inner filter effect is still absent (figure 1A). An increase of dF/dt in the beginning of the experiment can only be attributed to an increase of temperature. In case of a constant temperature, the shape of the diagnostic plot could be described by a decreasing branch of a parabola (for the rationale behind this see ref[37]).

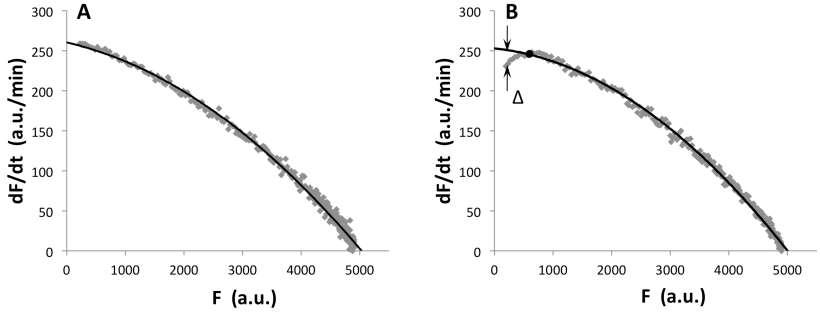

Figure 1. Diagnostic plots for the calibration curves measured after 15 min of preheating (A) and after 2 min of preheating (B).
Bold black point in section (B) corresponds to the end point of the "shaft" of the hockey stick. Δ is the difference between measured initial value of dF/dt and the value obtained from the extrapolation of the "shaft" to F_0.

The "hockey stick" is not a very precise metaphor here as the "shaft" is not straight, but we will still use it for the sake of visualization of the algorithm used for the evaluation of the magnitude of temperature changes.

When preheating of the samples is insufficient, it results in diagnostic plots with a characteristic "hockey stick" appearance (figure 1B). The initial uprising part of such a curve, representing the temperature relaxation process, corresponds to the "head" of the "hockey stick" and the descending part corresponds to its "shaft". The "hockey stick" is not a very precise metaphor here as the "shaft" is not straight, but we will still use it for the sake of visualization of the algorithm used for the evaluation of the magnitude of temperature changes.

For each diagnostic plot its part unperturbed by the temperature relaxation processes, i.e. the "shaft" of the hockey stick. The program selected the longest possible "shaft" on the diagnostic plot basing on the criteria that its initial point should lie inside standard deviation range of the residuals of a parabolic approximation fitted to the "shaft". Afterwards the parabolic approximation of the "shaft" was extrapolated backwards to the initial zero time point to calculate the difference (Δ) between measured initial value of dF/dt and the value obtained from the shaft's approximation (figure 2B). As an estimate of the temperature error we used the increase of the CF (CF_{inc}) at the zero-time point calculated as the ratio of Δ to the value of dF/dt measured at the beginning of the experiment:

$$\mathrm{CF_{inc}} = \frac{\Delta}{\frac{\mathrm{dF}}{\mathrm{dt}}\Big|_{t=0}} \qquad (1)$$

Finally, for each set of TG curves measured in the same plate the quality of temperature control was estimated by the averaged value of CF_{inc} calculated for all calibration curves present in that particular plate.

Evaluation of errors induced by calibration

During a TG experiment α2M-T forms from the interaction of the inhibitor α2-macroglobulin (α2M) with thrombin. α2M is present in sufficient excess over thrombin, ~3 vs. 0.3 µM respectively to safely assume that the reaction is first order. The amount of α2M-T formed is therefore directly proportional to the amount of thrombin and to the time it is active i.e. the final α2M-T concentration is proportional to the ETP. The ratio of the α2M-T end level to the ETP therefore is in theory constant and should be independent of any error that could be introduced by calibration.

We used this fact to remove the effect of possible calibrator activity errors. To this end we used the values of α2M-T_{end} as calculated by the Thrombinoscope® software to compare the individual α2M-T_{end} value to the average of all α2M-T_{end} values from the experimental data from the different laboratories combined. This comparison yielded a correction factor (R) that was applied to calculate an internally calibrated ETP (ETP_{ic}). So: $ETP_{ic} = R \cdot ETP_{exp}$ where R = (Average α2M-T_{end}) /

(Experimental α2M-T_{end}). An analogous formula was used to calculate "internally calibrated Peak".

Results

Continuous calibration, calculation of free thrombin

As explained under methods, it is possible to correct for calculation errors in the ETP via the ratio between the ETP and the end-level of α2M-T, because the proportion, to which ETP is over- or under-estimated by the calibration procedure, is the same as the proportion to which the final level of α2M-T is under- or overestimated. This property we used to correct the values of ETP and peak as described under methods. In table 2 it is seen in how far the experimental error is diminished by this procedure. In fact, the CV for the internally calibrated ETP is reduced by almost 30% as compared with the initial CV with a reduction from 16,3% to 11,7%. A similar trend is observed for the thrombin peak in which the inter-laboratory CV is reduced from 17,4% to 11,9%. The remaining error must be the experimental error in the curve of amidolytic activity, $f_a(t)$, which includes the error due to insufficient temperature equilibration and possible errors in the calculation of ETP and α2M-T from the raw data[38].

Errors due to insufficient temperature equilibration

For each experiment, the degree of the temperature control quality CF_{inc} was calculated. The corrected values of ETP and Peak for all 34 experiments are plotted against CF_{inc} (figure 2 C, D). The higher the temperature jump in the beginning, the lower the actual temperature was during the experiment. TG increases when the temperature decreases as can be seen when compared to figure 2 A, B; it is therefore not surprising to find higher TG values in the cases where there was a higher temperature jump. Figure 2 confirms this relation between TG parameters and CF_{inc}. Moreover, this relation allowed us to subtract a temperature dependent trend from the data and evaluate the variability of the data after this second correction.

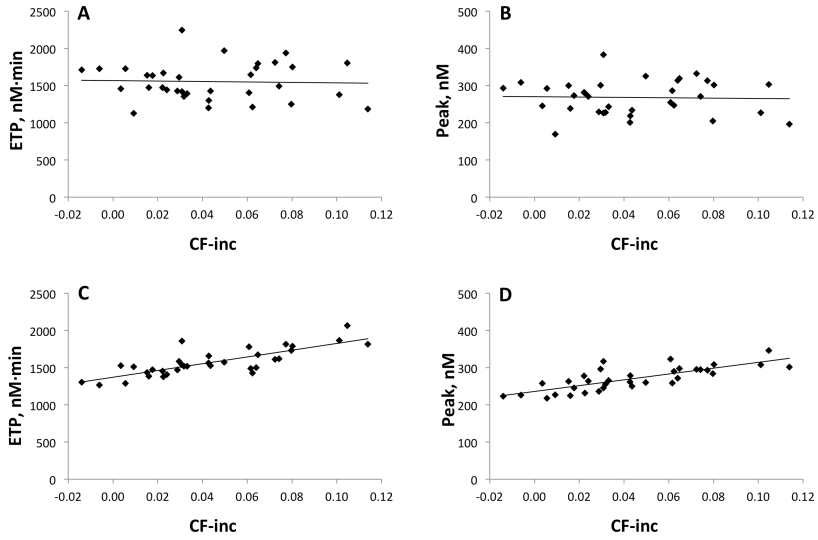

Figure 2. ETP and Peak versus temperature control estimation parameter CF-inc. (A, B) – initial values; (C, D) – values obtained after the elimination of calibrator activity errors.

Moreover, this relation allowed us to subtract a temperature dependent trend from the data and evaluate the variability of the data after this second correction.

Mean values, standard deviations and variation coefficients for corrected data are presented in table 2. In total the two successive correction procedures both for temperature and for calibration reduced the variability of ETP and Peak by nearly 50%.

Table 2. Mean values, standard deviations and variation coefficients for ETP and Peak, effect of temperature and calibration correction.

	ETP			Peak		
	initial	i.c.	i.c.+t.c.	initial	i.c.	i.c. + t.c.
Mean	1553.7	1571.0	1371.0	268.0	270.5	236.0
Standard deviation	252.8	183.5	111.3	46.5	32.3	20.3
Variation coefficient, %	16.3	11.7	8.1	17.4	11.9	8.6

Discussion

Normalization

Normalization seems to be an effective way to reduce the inter-laboratory variation for the assay[31,33,35,39]. However, it does not seem capable to correct for all different parameters under all different conditions and the remaining variability is still relatively high ~10%[39]. Normalization could therefore only present a partial solution to the inter-laboratory variation for further clinical implementation, since its major drawback results in the persistence of the error in both the normal and test sample, which reduces both the sensitivity and specificity of the assay. Lastly, since normalization adds experimental conditions (as well as costs and time) to the assay by testing the reference plasma this may further increases the barrier for clinical implementation of TG.

Calibration

The calibration procedure is necessary to relate observed reaction velocities (dF/dt) to thrombin concentrations i.e. to quantify the amount of thrombin that is formed during an experiment. The classical approach to calibration, is to determine a CF once and use this value for all subsequent time points and experiments. In TG experiments this approach is not feasible, as during the experiment the inner filter effect and substrate consumption continuously increase the CF. Therefore, at every level of fluorescence another CF applies. In the experiments shown here the CF increases by 45% from the beginning to the end of the experiments. Moreover, the lack of stability of the excitation beam gives erroneous fluctuations of the signal. These could also lead to discrepancies between a predetermined CF and the measured TG signal. Lastly the colour of the plasma leads to a difference in the signal intensity since there are plasma constituents that absorb the energy of the emission wavelength which differ from sample to sample.

In the experimental procedure, the course of total amidolytic activity $f_a(t)$ is found, consisting of free thrombin $f_g(t)$ and thrombin bound to α2M. Because the velocity of formation of α2M-T in the pseudo first order reaction α2M + T $\rightarrow$ α2M-T is proportional to the concentration of free thrombin, with the reaction constant k as the proportionality constant, the total amount of α2M-T formed is k times the integral of the thrombin-time curve.

The contributions of T and α2M-T apart thus can be calculated by numerical solution of the differential equation $f_a(t) = f_g(t) + k\int_0^t f_g(t)dt$. In the end ($t \rightarrow \infty$) $\int_0^\infty f_g(t)dt = ETP$ and $k\int_0^\infty f_g dt = k \cdot ETP$ is the end level of α2M-T.

In this procedure, the correct k is that one that allows the boundary condition that free thrombin becomes zero without ever having been significantly negative. In practice, this means that the point in time has to be estimated at which all free

thrombin has disappeared. Various strategies can be employed that need not be discussed here; suffice it to say that the ideal solution has not yet been found. The essential point is that if *k* is underestimated, part of the activity of α2M-T is attributed to free thrombin and contributes to the ETP, whereas when *k* is overestimated part of the free thrombin is attributed to α2M-T. Variations in the estimation of K might contribute to the residual error after compensation for temperature and calibration effects. Improvement in reliability of the estimation of K could be achieved by mathematical smoothing of the raw data prior to analysis.

Further reduction of the variability caused by calibration of the experiment can be sought in the internal calibration of the sample; for example, by utilizing the α2M-T that is formed during the experiment. However, solutions that correct for the colour of the plasma and the inner filter effect have still to be found in order to be able to quantify the thrombin concentration in the sample in a reliable way.

Temperature

The variation that is introduced by temperature differences throughout the course of the experiment is at large an operator error. If the sample temperature is meticulously tuned to the temperature inside the fluorometer, and/or if the software in the fluorometer enforces the operator to pre-heat the sample long enough, the causes of the “hockey stick” disappear. A problem of uniformity of temperature within the instrument remains. We observed differences of up to 2^{o}C in the same plate (manuscript in preparation). This suggests that for this type of experiments fluorometer should be equipped with more adequate temperature control. Currently samples are heated using air, which is typically known to be an insulator, if a more efficient conductor is used e.g. aluminium, the incubation time can be shortened to 5 min (manuscript in preparation). Which is more or less the same time that it takes to prepare the machine prior to the measurement, making the pre-heating step redundant.

Recommendations

Taking into account our current knowledge it stands to reason to perform a similar study as[35] to determine how the suggested improvements to the CAT hold up in "real life" conditions. An improved inter-laboratory variability could expedite the clinical implementation of this versatile method.

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Chapter 5

The Influence of Temperature on Thrombin Generation

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Manuscript in preparation

Abstract

Background Calibrated automated thrombography (CAT) is a global haemostasis assay and its outcome correlates well with bleeding and thrombosis risk. The assay itself has been proven to be highly sensitive for temperature, making temperature control of the experiment a necessary precondition for reproducible results. Temperature fluctuations throughout the experiment as well as between the wells in the plate have been shown to add to the variation in the results.

Objectives In this study we aim to identify and where possible quantify the influence of temperature on thrombin generation (TG). Moreover, by comparing the difference in response between defibrinated normal pooled plasma (NPP) and regular NPP we aim to further elucidate whether the control mechanisms of thrombin formation are diffusion limited.

Methods TG was measured at a range over temperatures from 30.5°C until 38.5°C with 2°C intervals in NPP and defibrinated NPP. The TG parameters where calculated using the new insights presented in chapter 6. Prothrombin conversion and thrombin inactivation were assessed.

Results The TG parameters in NPP plasma where decreased at increasing temperature -3.2%/°C for the peak and -4.4%/°C for the ETP. Defibrinated plasma showed no temperature response for the peak and a -1.4%/°C decrease for the ETP. The calibration reaction increased with temperature by 3.8%/°C.
No anomalies have been observed as a result of the lower substrate concentration.

Conclusions TG parameters are decreased at increasing temperatures as a result of the difference in response of the pro- and anticoagulant processes of TG. Calibration of the TG increased the response to temperature changes in the TG parameters peak and ETP as a result of the opposite response to temperature between TG and calibrator.

Introduction

Coagulation assays, the Calibrated Automated Thrombogram (CAT) as described by Hemker[1] being no exception, try to mimic the human physiological conditions as closely as possible. We all understand that concessions have to be made, because we cannot (as yet) represent the full physiology of the human body in in vitro coagulation tests. However, the one physiological aspect that most of the test really stringently adhere to is the representation of the human core body temperature, which is generally excepted to be around 37°C with a healthy state being considered to be between 36,1°C and 37,2°C[2].

Coagulation is a highly complex mechanism that can easily be influenced by many factors one of these being temperature[3,4]. As a consequence, perfect thermostability is a *condition sine qua non* for reproducible measurement of thrombin generation (TG). It is therefore almost intuitively that we perform the test at 37°C. Unfortunately, as is shown in chapter 4, temperature also happens to be one of the sources of error of the test results of TG and therewith one of limiting factors of comparison of results between laboratories.

The control of temperature in any device is often taken for granted and is generally considered to be the responsibility of the manufacturer. For the CAT it has been shown that both the instability of temperature throughout the 96 well plate[5] as well as the temperature throughout the thrombin generation (TG) experiment[3] influence the outcome of the TG experiment. It is not the purpose of this chapter to further elaborate on the technicalities of temperature control, but since they are important to understand, they are briefly presented in an appendix to this chapter, therewith accompanied by a brief description of an amelioration for the CAT to improve temperature stability and control.

TG is the result of prothrombin conversion and thrombin inactivation. If both processes would have approximately the same temperature coefficient, TG would be largely temperature independent and temperature control requirements would be easily met. Previously it has been shown, however, that lowering the temperature increases thrombin generation[4]. The assumption was that prothrombin conversion in the presence of fibrin is diffusion limited and therefore much less sensitive to lowering of temperature than thrombin inactivation[4], which, like most (bio)chemical reactions will decrease its velocity (in a very rough approximation) by a factor two for every 10°C drop in temperature. Oddly enough, this observation is not supported by the clinic where hypothermia (<32°C) in trauma patients, is associated with an inadvertent bleeding risk[6].

In the CAT method thrombin concentrations are calculated by comparing the fluorescence in the well where thrombin generates to that of the fluorescence that is generated in the calibrator well, where a known amount of $\alpha_2$macroglobulin-thrombin (α_2m-T) complex converts the same substrate under the same experimental conditions (3). A difference in the temperature between the calibrator and TG well would therefore lead to either an over or underestimation of the thrombin concentration.

In order to investigate the extend of the influence of temperature disturbances on thrombin generation, we took a more fundamental approach by performing TG experiments at different temperatures. These experiments give us a good understanding of how the enzymatic reactions of the blood coagulation system respond to changes in temperature. And allow us to extrapolated and speculate on the influence of the more stochastic temperature fluctuations during the TG experiment.

Material and methods

TG was measured in normal pooled platelet poor plasma (PPP) at 5 pM tissue factor (TF). A bespoke temperature controller was built into the fluorometer. It consists of a PID controller that steers a resistive heater attached to an aluminium mould. The aluminium decreases the overall temperature variation throughout the 96 wells plate to < 0.1°C. The appendix to this chapter can be consulted for further information on the technical details behind the construction of the temperature controller.

Fluorescence based measurement of thrombin activity

The development of fluorescence intensity from 7-Amino-4-methylcoumarin (AMC) was measured in a 96-well plate fluorometer (Ascent reader, Thermolabsystems OY, Helsinki Finland) equipped with a 390/460 filter set (excitation/emission) and a dispenser. Immulon 2HB, flat-bottom 96-well plates (Dynex) are used. six readings are done per minute and experiments have been carried out in quadruplicate unless otherwise indicated. Per temperature two separate runs of experiments have been performed.

To each well, 80 µL normal pooled plasma is added. Wells in which TG is measured receive 20 µL of buffer I, containing 30 or 600 pM of recombinant tissue factor (TF) and 24 µM phospholipid preparation. Wells in which constant thrombin-like activity is measured receive 20 µL of the α2M-thrombin (α2M-T) solution which has a thrombin-like activity of 600 nM, resulting in a final concentration of 100 nM thrombin like activity in the well. Thrombin generation (TG) was initiated by the addition of 20 µl of ZGGR-AMC (150 µM) and $CaCl_2$ (16.6 mM).

The dispenser of the fluorometer is flushed with mili q water, emptied and then flushed with a prewarmed solution of the substrate and calcium in buffer II (referred to as FluCa) as stated in the Thrombinoscope manual. The "FluCa trigger" solution, a mixture of fluorogenic substrate and $CaCl_2$ is prepared as follows. To 1782 µL of buffer B, at 37°C, 200 µL of 1 M $CaCl_2$ is added, then 18 µL of a DMSO solution of 50 mM ZGGR-AMC is squirted in and immediately vigorously mixed on a vortex. The pre-heating temperatures of the dispensing liquids have been adjusted to the measurement temperatures accordingly. At the start of the experiment, the instrument dispenses 20 µL of the substrate/calcium mixture to all the wells, registers this as zero time, shakes them for 10 s and starts reading.

Reagents

Innovin recombinant human Tissue Factor (rTF) was used at final concentrations of 5pM and 100pM (Dade-Behring, Marburg, Germany). The calibrator (α_2m-T) was prepared as previously described by Hemker et al.[7]. Procoagulant phospholipids (PL), containing 60% dioleoyl PC, 20% dioleoyl PS and 20% dioleoyl PE, were prepared as described by[8]. ZGGR-AMC was purchased at Bachem (Basel, Switzerland) and dissolved in DMSO to a stock concentration of 50 mM.

Buffers

Buffer I used in the preparation of dilutions of the reagents: 20 mM Hepes, 140 mM NaCl, 0.02% NaN_3 and 5 mg/mL BSA at pH 7.35.
Buffer II Used for the solution of the fluorogenic substrate: 20 mM Hepes, 0.02 % NaN_3 and 60 mg/mL BSA at pH 7.35. The buffers are filtered using a corning filter system 250 mL with 0.2 µm PES membrane and stored at -20°C.

Plasma preparation

Blood was obtained through antecubital venepuncture (1 volume trisodium citrate 0.13 M to 9 volumes blood) in a 10 ml vacutainer tube (BD, San Jose CA, USA) from apparently healthy volunteers after agreement to informed consent in concordance with the declaration of Helsinki. The blood was centrifuged twice at 2,821g at room temperature for 10 minutes (min). Platelet-poor plasma was collected, and a pool (NP) was prepared and stored in 1 ml containers at –80°C.

Defibrination of normal pool plasma

Plasma was defibrinated using 1 U/ml ancrod (NIBSC, South Mimms, UK). The dried ancrod powder was dissolved in milli q water so that 17 µl could be added to 983 µl of citrated normal pool plasma to get the 1 U/ml final concentration. A clot was allowed to form during a 10 min incubation at 37°C, and a subsequent 10 min incubation on ice. The fibrin clot was removed by winding it on a plastic spatula.

Data handling

The raw data of optical density or fluorescence measurements of thrombin activity were exported to Excel (Microsoft Excel 2003) and corrected for substrate consumption and non-linearity of fluorescence with the concentration of the fluorophore by the method further described in chapter 6 of this book. The thrombin decay constants and the subsequent curves for prothrombin conversion and thrombin decay where determined according to the method of Kremers[9].
The TG parameters used to represent the temperature effects are limited to the Calibrator level, the PEAK and the ETP. The Lagtime and Time to Peak are not included as they give an incomplete overview of the action of thrombin over time and are therefore considered to be less representative to elucidate the full scope of the influence of temperature on TG.

Results

Temperature dependencies

In the first column of tables 1 and 2 the temperature dependency of the calibration reaction. both in NPP (table 1) and in defibrinated NPP (table 2), are shown. The average increase in calibrator activity is around 4% per 1°C, with little more increase in the calibrator from 36.5°C to 38.5°C. This differs from the temperature dependencies of the TG reactions that are shown in columns 2-5 of tables 1 and 2, moreover there are also distinctive differences between the NPP and the defibrinated NPP experiments

The TG activity is represented here by the PEAK and the ETP values, in the NPP both values decrease with an increase of temperature, table 1. columns 2 and 3. The PEAK value decreases with an average of 3.2% per °C and the ETP with 4.4% per °C. No stagnation of the decrease of Peak and ETP values is observed from 36.5 to 38.5°C. The decrease of PEAK and ETP values is aggravated when the data are multiplied with their respective calibration factors, table 1 columns 4 and 5. This can be explained by the opposing temperature trends between the TG and the calibration experiments, this causes the lowest temperatures to be corrected less and the highest more.

Table 1: Temperature dependence of the TG experiment from 30.5°C until 38.5°C measured in NPP.

Temp °C	Calibrator slope	dF/dt		Thrombin	
		Peak Max FU	ETP FU*min	Peak nmol/L	ETP nmol .min/L
	Average	Average	Average	Average	Average
30.5	145±1.9	310±2.5	1568±12.5	224±1.8	1140±9.1
32.5	163±6.4	297±5.6	1409±26.7	187±3.5	892±16.9
34.5	176±3.7	277±3.4	1301±16.1	163±2.1	772±9.5
36.5	185±2.6	268±6.5	1175±28.5	145±3.5	639±15.5
38.5	186±2.7	225±4.4	1039±20.2	120±2.4	552±10.7
(%/°C)	+3.8	-3.2	-4.4	-4.6	-5.6

Table 2: Temperature dependence of the TG experiment from 30.5°C until 38.5°C measured in defibrinated NPP.

Temp °C	Calibrator	dF/dt FU		Thrombin	
	Slope	Peak Max FU	ETP FU.min	Peak nmol/L	ETP nmol.min/L
	Average	Average	Average	Average	Average
30.5	154±2.3	175±2.7	730±11.1	125±1.9	589±8.9
32.5	167±7.1	167±4.7	683±19.3	106±3.0	495±14.0
34.5	190±3.1	169±2.9	680±11.5	97±1.6	448±7.6
36.5	200±2.1	171±2.5	678±9.9	91±1.3	399±5.8
38.5	205±1.9	163±2.8	652±11.3	83±1.4	337±5.8
(%/°C)	+3.8	+0.001	-1.4	-2.8	-4.5

In defibrinated NPP the effect of a diminishing Peak and ETP is much less pronounced with no decrease of the Peak and a 1.4% per 1°C decrease of the ETP, table 2 columns 2 and 3. Again this effect is amplified by the calibrator table 2 columns 4 and 5, where the multiplication with the calibration factor enhances the effect to 2.8 and 4.5 % per 1°C decrease for the Peak and ETP respectively. The Lag-time in both instances, NPP and defibrinated NPP, decreases with increasing temperature from 1.16 min at 30.5°C to 0.83 min at 38.5°C, suggesting a more procoagulant state at higher temperatures.

An approximation of the decay constants for NPP and defibrinated NPP can be found in table 3 an increase in the constants can be observed with increasing temperature.

Table 3. Approximated decay constants for TG experiments at a range of temperatures

Temperature	Decay constant defibrinated NPP	Decay constant NPP
30.5	-0.71	-0.42
32.5	-0.85	-0.49
34.5	-0.90	-0.56
36.5	-1.04	-0.63
38.5	-1.04	-0.72

Figure 1 illustrates the prothrombin conversion curves that have been obtained using the method by Kremers et al.[9] and the decay constants from table 3. It shows an overall slight increase in prothrombin conversion with time. In defibrinated NPP, figure 2, the increase in prothrombin conversion is more pronounced.

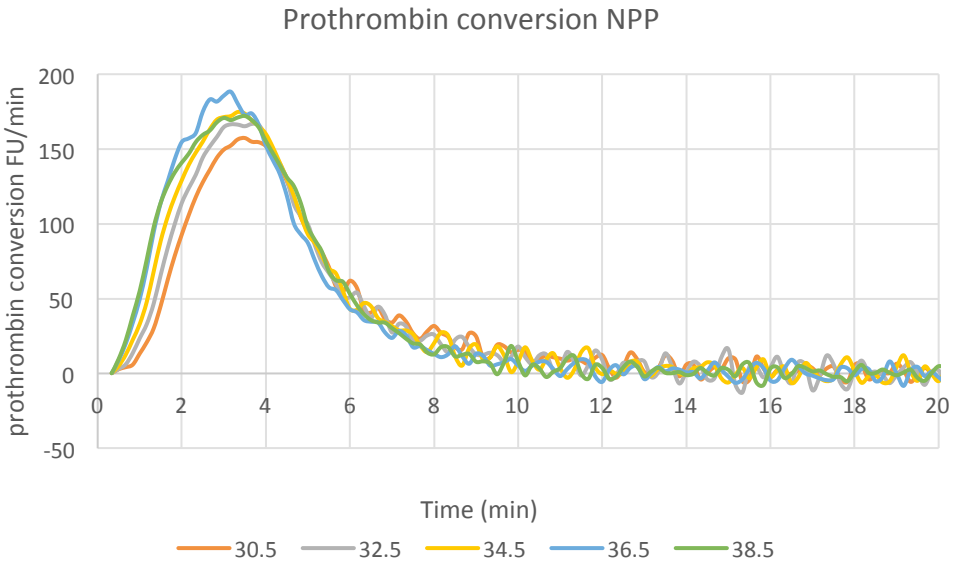

Figure 1: approximation of prothrombin conversion in NPP measured at 5pM TF.

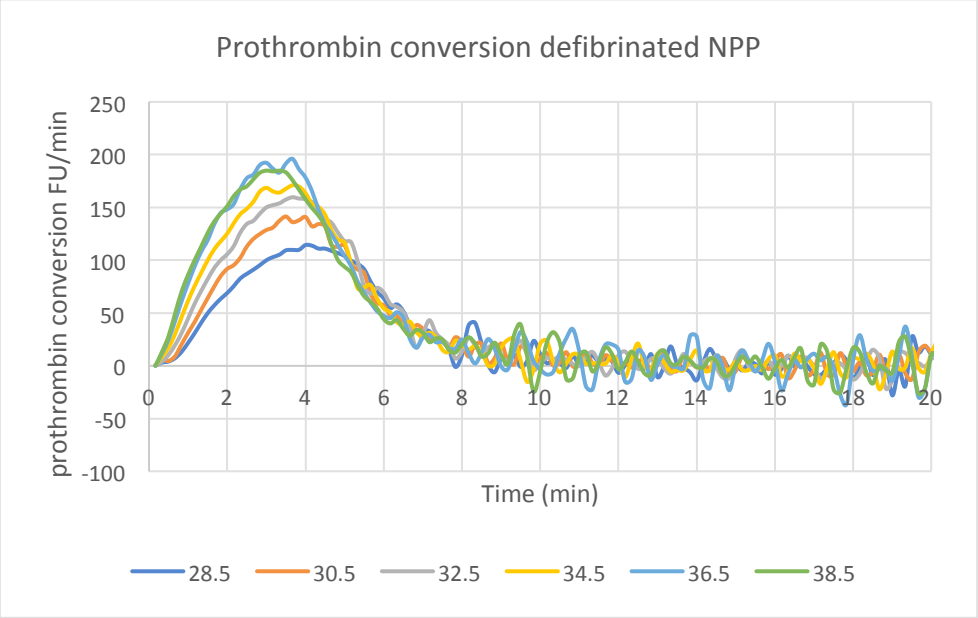

Figure 2: approximation of prothrombin conversion in NPP measured at 5pM TF.

Substrate concentration

The maximum level of fluorescence that was attained in these experiments with a substrate concentration of 150 µM was 3200 FU. The highest level of fluorescence over the functional part of the TG curve was measured at 2000 FU for the 100 pM TF concentration at 28.5°C.

Discussion

Humans are homeothermic (warm blooded) species, they generally have a core body temperature of 37°C. Several mechanisms of heating and cooling are applied to sustain this steady state thermal equilibrium. These mechanisms combined with the irregular geometry of the human body, lead to a complicated three-dimensional temperature distribution. This causes a temperature gradient that is not just longitudinal but also axial. Moreover, this gradient is greatly influenced by the outside temperature. To assume that 37°C is therefore the most representative temperature to measure coagulation is by itself up for debate.

Heat loss is, in part, regulated through conduction in the peripheral blood flow. The large vessels that lie deeper in the extremities are in parallel with one another.

This allows the warmer outward flowing arterial blood to give off heat to the inward flowing venous blood. This so-called counter-current principle causes a steeper axial temperature gradient. When thrombosis, that is often found in veins of the lower legs, is considered, it just as likely as not that it occurs under lower temperatures <35°C then at 37°C. And if the temperature was lower, a different dynamic of the coagulation system led to its formation.

Moving beyond the temperature differences it can be further narrowed down to the site of an injury where a temperature gradient can very well be expected between the underlying tissue and the ruptured skin and vessels that are exposed to the air. Heat loss of the blood and tissue as they are exposed to air is very likely to be expected at the site where the plug has to be formed to halt bleeding.

Our results show that thrombin generation increases considerably when the temperature drops. In the 30.5-38.5°C interval the ETP increases by nearly 5.6% per degree and the peak by 4.6%. Thereby providing a possibly explanation for the degree of variation that can be a result of a temperature gradient within the plate and/or throughout the experiment.

The increase of thrombin generation with decreasing temperature is much more apparent in the presence of a fibrin web, this contradicts the previously made assumption that in the presence of a fibrin web prothrombin conversion is diffusion limited and therefore less temperature dependent than thrombin inactivation, which is a bi-molecular reaction in free solution[4]. However, the prothrombin conversion curves and decay constants illustrate that both prothrombin conversion and thrombin decay increase with increasing temperature. The overall result of a decrease in TG with increasing temperature can therefore only be explained by the fact that the procoagulant reactions are influenced less by temperature than the decay reactions. This again hints to the possibility of diffusion limited reaction rates in thrombin formation but these results are however not conclusive enough to either confirm or reject the previously posed hypothesis in the work by Hemker et al.[4].

The contradictory results of the TG with lowering temperatures are a good example of the surplus value of measuring beyond the formation of a clot. Whilst clot formation i.e. the clotting time are delayed with decreasing temperature, overall TG as represented by the PEAK and the ETP are increased, indicating a more prothrombotic phenotype. Interestingly it is believed that bleeding is a result of hypothermia in trauma patients, the idea that this bleeding is amongst other things a result of delayed reaction rates of the clotting enzymes is supported by clotting times [6] but now contradicted by TG results. The result of the differences between these reactions in NPP is that the overall lower amount of thrombin is

able to cleave more substrate over a longer time at lower temperatures where it is cleared less rapidly relative to its formation.

The differences in the influence of temperature between the TG- and the calibrator well and deviations from the standard (37°C) temperature will influence the results of a CAT experiment. A multiplication by a calibration standard that was also under the influence of the same temperature fluctuation can therefore aggravate the variation that is observed in either of the two parameters alone.

Being the first series of experiments to be performed with a 150 µM substrate concentration, albeit out of necessity, no adverse consequences where observed throughout the experiment. Moreover, the maximum fluorescence of the functional part of the TG curve never surpassed more than 60% of the maximum fluorescence attainable for this substrate concentration in any of the experiments. The defibrinated samples do reach the maximum fluorescence over the course of 60 min that the experiment is run. However, this is due to the large contribution of α2M-T, that clears a larger portion of thrombin in the absence of fibrin, the relevant part of the TG curve has finished long before this value is reached. Therefore, the most important precondition for the calculation method that was used and that will be further elaborated upon in chapter 6 still seems to be fulfilled.

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This chapter is embargoed at request

Chapter 6

The relation between thrombin concentration and reaction velocity as measured from the conversion of fluorogenic substrate.

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Manuscript in preparation

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Chapter 7

Indirect Measurement of Initial Reaction Rates from Progress Curves, its Application to Investigate the Influence of Albumin on the Interaction of Thrombin and the α2macroglobulin-Thrombin Complex With the Fluorogenic Substrate ZGGR-AMC.

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Chapter 8

Correction of the Effect of the Fluorogenic Thrombin Substrate Used in Calibrated Automated Thrombography on Physiological Thrombin Generation.

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Manuscript in preparation

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Chapter 9

General Discussion and Conclusions

The global hemostatic assay that measures thrombin generation (TG) in plasma increasingly gained attention as a diagnostic tool in the field of thrombosis and hemostasis. The Calibrated Automated Thrombin Generation test (CAT-TG)[1] has been used in research laboratories for over 15 years now. And has over this period of time been implemented in over a thousand research laboratories worldwide.
Throughout the last decades considerable evidence has been published that the amount of thrombin activity measured with TG is an indicator of the risk of bleeding[2–5] as well as venous thrombosis[6–14]. TG can be used to distinguish mild from severe bleeding phenotypes in hemophilia A patients with comparable factor VIII levels[15]. It also relates to the risk of arterial thrombosis, be it in a less straightforward manner[16–18]. It is a common denominator of the effect of all anticoagulants[19–26] and, when it is measured in platelet rich plasma, it is inhibited by platelet inhibitors[27,28]. Therefore, it has a promising outlook for use as a clinical parameter. Nevertheless, its implementation in the clinical laboratory until this moment is very limited.

One reason is that the CAT-TG is not a fully automated method like most other methods in the clinical routine laboratory. It still requires an amount of old-fashioned laboratory labour. Apart from the fact that this is not in line with modern laboratory management, it also introduces the executor as a cause of inter-laboratory variability. Strict training of the technicians is a sine qua non for obtaining acceptable results (Prof. Y. Dargaud, personal communication).

Another way to introduce TG in the clinic is by so called near patient testing or point of care (POC) testing[29–31]. In chapter 2 we described the development of a near patient, miniaturized device based upon a previously published method, developed in our laboratories, that allows the measurement of TG in whole-blood[32]. Measurement in whole blood is necessary anyhow because obtaining plasma by centrifugation is hard to realise in a point of care situation. Anyhow, a full TG-curve requires about 15 minutes, which is long if an immediate answer is required as e.g. in emergency bleedings.

To further decrease the turnover time an alternative calculation method is presented in which only the data until the peak of the TG are used. Although much information present in the total TG is thus discarded, it can be argued that both pro- and antithrombotic processes contribute to the peak value and the area under the TG curve up to the peak value. In how far this information is useful and sufficient remains to be determined for each individual pathological condition. One can imagine e.g. that it suffices as a quick estimate of the effects of vitamin K antagonists and heparin but that it will do worse for direct inhibitors of factor Xa, due to the blunted form of the TG-peak that such anticoagulants induce.

In chapter 2 we show that we have been successfully able to miniaturize the device to have a similar inter-assay variation as the original test. The fact that only one sample can be measured at any time excludes determination of the intra-assay variation. Also, the impossibility of a simultaneous control step will increase the chances of introducing errors. Nevertheless, a comparison of the results obtained with the adapted calculation method to the standard shows decent correlation coefficients 0.868, 0.998, 0.993 and 0.981 for the (peak)ETP, peak, lag-time and time-to-peak, respectively. The imperfect correlations are most likely due to the fact that the information from the decay phase of thrombin generation is discarded.

Clinical validation of this test has been performed on a group of 33 patients that underwent cardiothoracic surgery, with the aim to check whether the test would predict the degree of blood loss. After division of the patients into two groups (severe and mild bleeders) based on the mean drainage volume, a statistically significant difference was found between the two groups for the peak-ETP and the peak. This does not proof that the test can be used for predictive purposes but it is indicative of a statistically significant association. In the designing of this experiment, we had to trade of between the desire to have a homogenous sample population and a large number of samples. We have chosen for the first and restricted the type of intervention to Coronary Artery Bypass Grafting (CABG). This caused the sample size to be small and leaves room for more extensive studies in the future.

Simultaneously with the development of the near patient TG device, we wanted to make use of the miniaturised set-up to explore possibilities of alternative applications of TG. The zebrafish being a more and more used model, notably in fundamental studies on the relation between the genome and anatomy and physiology, we wanted to investigate whether this animal also could be used in experiments on coagulation physiology.

In chapter 3 we show that by applying the miniaturised model to zebrafish we are able to generate TG curves with an acceptable inter-individual variation with a mean CV of 14%. Moreover, we were able to detect a dose-response effect of two anticoagulant drugs: Rivaroxaban (a direct FXa inhibitor) and Melagatran (a direct FIIa inhibitor). Despite an encouraging start, the lack of physiological similarity between zebrafish and humans has led us to decide not to continue this path any further at this moment. Nevertheless, we believe that being able to measure extremely small quantities of blood in such frail animals brings many opportunities for future animal studies.

After this brief animal excursion, we understood that the miniaturized version, that we derived from the whole blood-TG method was in its current form not the way to quick clinical application.
We realized that one of the major drawbacks in the method remained the, relatively large, inter-laboratory variability and bad standardization criteria[33–36] which are only partly ameliorated by normalization[33–35,37]. Notably in the case of (extreme) hypo coagulability normalization is not able to reduce the inter-laboratory variation. As the matter of fact normalization actually caused an increase in variation of some of the parameters[33].

We were in the lucky circumstance that, through the cooperation with a number of clinical labs, we could lay our hands on the raw fluorescence curves from a reproducibility study carried out in France. Analysis of these curves allowed us to gain insight into the reasons for the large interlaboratory variability (Chapter 4). The biggest contributor to the total error appeared to be the comparison between the TG-sample and the calibrator. We were able to assess the contribution of the calibrator to the total error by making use of the ratio between the ETP and the end-level of α_2M-T. This can be done because the proportion, to which ETP is over- or under-estimated by the calibration procedure, is the same as the proportion to which the final level of α_2M -T is under- or overestimated.

By applying this correction, the inter-laboratory CV for the ETP could be reduced from 16,3% to 11,7%. A similar trend is observed for the thrombin peak in which the inter-laboratory CV is reduced from 17,4% to 11,9%. It thus appears that the calibration step, that has been designed as a tool for quantification and elimination of the known (inner filter, substrate consumption and plasma colour) and unknown measurement artefacts, in fact contributes significantly to the experimental error. This phenomenon can, hypothetically, be attributed to two reasons: The first is that in any procedure in which the data from two observations (calibrator and TG-curve) are combined, the experimental error in the outcome sums-up. The second being that the calculations used to combine the outcomes introduce systematic errors.
As a second major source of error we could identify the inconsistency of temperature, in particular as a result of inadequate pre-heating of the sample. We therefore had a closer look into both temperature control and calculation methods. In chapter 4 we re-introduce the “diagnostic plot” as a tool to determine temperature stability at the start of the experiment. The diagnostic plot is a plot of the first derivative of the fluorescence curve against the value of the fluorescence itself [38,39].

In a calibrator experiment performed at constant temperature, dF/dt is at its maximum at zero time because both the substrate concentration is highest and

the inner filter effect is still absent. An increase of dF/dt at the start of the experiment can therefore only relate to an increase of temperature. The characteristic “hockey stick” shape of the first derivative of the calibrator curve can only indicate a rise in temperature at the start of the experiment as there is no other explanation for the increase in reaction velocity. This error is mainly due to the operator that has not pre-heated the sample to the exact value of that in the fluorometer. Design changes to the software that enforce a fixed pre-heating time as well as creating operator awareness of the importance of temperature could partly improve the situation, be it that the time required to attain the right temperature is longer than ten minutes and that evaporation of the sample begins to play a role then.

After having seen the important role of temperature in the CAT-TG and not having been able to find any conclusive literature on the topic of temperature and TG, we decided to investigate the influence of temperature further in systematic matter, not just as a source of error. In chapter 5 the results of a series of TG measurements that have been performed over a range of temperatures (30.5°C – 38.5°C) are presented. Since temperature control and stability of the device appeared not to be up to the required standards, it was decided that a technical amelioration imposed itself. On purpose the technical details are not discussed in chapter 5 but in an appendix, as they are of a very different nature than the rest of the chapter. It is nevertheless vital to illustrate the intricacies of temperature control to fully understand why the current method is not sufficient.

The influence of temperature on TG was measured in normal pooled plasma (NPP) and in defibrinated NPP, in order to find an answer to the hypothesis weather or not TG is diffusion controlled. The assumption was that prothrombin conversion in the presence of fibrin is diffusion limited and therefore much less sensitive to lowering of temperature than thrombin inactivation [40]. In reality the opposite seems to be true with the defibrinated plasma showing much less of response to temperature changes, +0.001 %/°C for the peak values and -1.4 %/°C for the endogenous thrombin potential (ETP). NPP plasma on the other hand is more influenced by temperature with a decrease of -3.2%/°C for the peak and 4.4%/°C for the ETP.

The observation that TG is decreasing with increasing temperature is counter intuitive as most (bio)chemical reactions increase with temperature. TG however consists of both pro- and anticoagulant process and as the data in chapter 5 illustrate the procoagulant reactions are less influenced by temperature as compared to the anticoagulant reactions. If both these processes would be influenced in the same way by temperature, TG would be temperature insensitive. However, the combination of a lower overall prothrombin conversion with an even

more decreased activity of the antithrombins explain a higher apparent thrombin generation. This difference in response between pro- and anticoagulant responses can potentially be explained by the original hypothesis of diffusion-controlled mechanisms of thrombin formation. Unfortunately, experimental constraints in determining accurate decay constants and the lack of sensitivity of the measurement do not allow to either support or falsify this hypothesis.
An important practical point is the influence of temperature on the calibration experiment, that differs markedly from that on thrombin generation. The velocity of substrate conversion by the calibrator increases with increasing temperature and unlike thrombin generation, that decreases. This increases the temperature dependency of the thrombin generation curve that results from the comparison with the calibrator.

Inspection of a calibration curve immediately shows that it is far from being a straight line. Because of this non-linearity the calibration factor that converts the observed reaction velocity into an enzyme concentration changes with the level of fluorescence. Two mechanisms are thought to cause this phenomenon: Firstly, the inner filter effect (IFE) which makes that the fluorescence intensity decreases proportionally to the increasing concentration of fluorescent product. Secondly, substrate consumption (SC), which causes the reaction velocity to decrease proportionally with enzyme concentration as the reaction proceeds. In the CAT-TG an algorithm is used that compares the fluorescence curve from the calibrator well with the TG well and corrects for IFE and SC (see[39]). This algorithm is not based on insight in the mechanisms (IFE and SC) and therefore could be a source of error. In chapter 6 these phenomena that cause the non-linearity of the calibration reaction of TG are assessed and a calculation method is presented based on the insight into the mechanisms.

The relation between the measured fluorescence (F_{exp}) and the concentration of the fluorophore (C) is known to be: $\mathrm{F} = ACe^{-bC}$ with F=fluorescence, C=concentration and A and b constants. We found that within the range of fluorescence that we encounter in our experiments this formula can be replaced by $F_{exp} = F_{max}(1 - e^{-kC})$. We show that the latter formula, in contrast to the first allows to develop a simple expression for the correction of the inner filter effect: $F_{ideal} = -F_{max}\, ln\left(1-F_{exp}/F_{max}\right)$ (Chapter 6). This formula is generally applicable and is based on the optics of the inner filter effect.

In any fluorometer of the type used for the CAT-TG, this formula can be used after normalization of the fluorescence intensity at 300 μM AMC to 1000 AU and using 2090 as the value of F_{max} or, alternatively, using 2.09 times the fluorescence intensity at 300 μM AMC for F_{max}. This operation will work as long as all other

variables of the lambert-beer equation ($A = log_{10}\left(\frac{I_0}{I}\right) = \varepsilon.I.c$) stay the same. Mind that the intensity of fluorescence at 300 μM is not a constant for a given fluorometer but changes with the intensity of the excitation light source, which diminishes with time. The fluorescence intensity at 300 μM AMC should therefore be regularly controlled.
The experiments presented in chapter 6 show that the conversion of substrate by α_2M-T can be described by the classical Michaelis-Menten hyperbola $\frac{dP}{dt} = E.k_{cat}.S/(Km + S)$, so that we can calculate the relation between reaction velocity and enzyme concentration as $E = (\frac{dP}{dt})(Km + S)/(k_{cat}.S)$, after the IFE has been corrected for with the abovementioned formula. This relation between reaction velocity and enzyme concentration is independent of a calibrator curve, the calculation only requires that the kinetic constants Km and k_{cat} are known.

The variation in the optical density of the plasma due to the difference in colour of individual plasmas can be as large as 10%, ignoring the plasma colour could introduce considerable experimental variation. Plasma colour being directly related to the optical density can however easily be corrected for with a simple multiplication factor.

The fluorogenic substrate that is used in TG is only soluble in a buffer containing high quantities of Bovine Serum Albumin this implies that the substrate binds to albumin. Given the fact that albumin is the most abundant plasma protein made us question how changes in albumin level could influence a TG- experiment. We therefore investigated the influence of albumin concentrations on the conversion of ZGGR-AMC by thrombin and by α2M-T in chapter 7. This involves measurement of the initial rates of substrate conversion in buffer with different concentrations of albumin. Classical enzyme kinetics requires measurements of initial rates, i.e. measuring in a time frame where substrate consumption is negligible. This however is precisely the time frame in which the measurement in a fluorometer of the type we used is instable. We therefore measured over the time frame in which the product curve is parabolic. A parabolic progress curve implies a linear relation between reaction velocity and substrate concentration. This is to be expected in the beginning of the reaction where the change of S in the numerator of the Michaelis-Menten equation ($E.k_{cat}.S$) does count whereas in the denominator ($Km + S$) it is still negligible because $Km \gg S$ The initial rate therefore can be calculated as the linear component in a parabola fitted to the first part of the fluorescence trace. This principle is applicable to the measurement of initial rates of calibrator curves in plasma as well.

We found that albumin has hardly an influence on the kinetic constants of thrombin but a large influence of the Km of α2M-T. This we explained by assuming that the substrate binds reversibly to albumin but that thrombin is capable of splitting the bound substrate whereas α2M-T is not.

Substrate concentration

In chapter 8 an assessment of the influence of the fluorogenic ZGGR-AMC substrate on the TG experiment was investigated in a concentration dependent manner. The presence of the substrate causes a fraction of the thrombin to be substrate bound to its active centre. Therefore, less thrombin is available to interact with its inhibitors antithrombin (AT) and α_2Macroglobulin (α_2M). Indeed, substrate was found to increase TG values because of this competition. Splitting the TG curve in a prothrombin conversion- and a decay-curve[41] showed that the main effect of the substrate is on the attenuation of TG by decreasing its half-life time. Naturally, occupation of the active center of thrombin by substrate not only slows down decay but also inhibits positive feedback effects like those on factors V, VIII XI and negative effects after binding of thrombin to thrombomodulin. This, theoretically, calls for using minimal substrate concentrations. The original choice for the concentration of the thrombin substrate was dictated more by solubility than any other consideration (chapter 7).

In chapter 4 we lowered the substrate concentration of the experiments to 150 μM and found no adverse effects on the thrombin generation curves. At this concentration no considerable effect of substrate depletion was observed as the end level fluorescence of the TG experiment was about 65% of the maximum fluorescence, like in experiments at higher substrate concentrations. It thus appears that substrate consumption is roughly proportional to the inhibition of thrombin decay by the substrate.

Now the question poses itself whether we can suggest amelioration of current practice of TG-measurement in the light of our results, either by adaptations that relate to calibration, to the fluorogenic substrate, to the calculation method and to technical ameliorations.

Calibration

Calibration is currently a necessity to be able to quantify the amount of thrombin generated and to correct for the specific colour of the plasma as well as for the inner filter effect and substrate consumption. The calibration experiment is run in parallel in a separate experimental well. Even at the highest level of scrutiny an increase of experimental error must be introduced by comparison of two experiments and the calculations that this involves. The most adequate solution for this would be to abolish the need for an "external" calibration experiment and internalize it. It has been suggested by Hemker (A self-calibrated thrombin generation assay. European patent application P6066184EP) that it is possible to use an “internal” ratio for the calibration. When a small fixed amount of product is added to the reaction mixture before the start of the TG reaction, and when the inner filter effect is compensated for so that fluorescence is proportional to

product concentration, then the progress of the reaction can be expressed as the ratio of the level of fluorescence to the initial level. $r_{exp} = ((F_{exp}/F_0) - 1)$. This already compensates for effects of the colour of plasma etc. Also, this allows to express the progress of the reaction in terms of the fraction of substrate consumed. From the present work we know that simple Michaelis-Menten kinetics apply, so it must be possible to calculate, at each level of substrate consumption, the amount of enzyme (thrombin) that causes the measured reaction velocity.

The fluorogenic substrate.

The substrate has a pronounced effect on the development of the TG curve as the substrate competes for thrombin with the natural substrates that are present in the plasma. We have shown the main effects to be in the thrombin decay phase and to a lesser extend in the thrombin formation phase. Moreover, we have also shown that it is possible to correct for the competitive effect of the substrate. Ideally a substrate with very high Km should be used of which only a small fraction is consumed. In that case Km+S remains practically constant during the experiment and the reaction velocity is simply proportional to enzyme concentration and substrate concentration, which, in combination with the ratio method mentioned above would allow simple direct determination of the TG-curve.
Substrates that are not split by the α2M-T complex have many advantages: less substrate will be consumed and the inner filter effect would be less important, as the overall fluorescent trace would remain relatively low. The calculation step required to eliminate the contribution of the α2M-T complex to the total amidolytic activity would become superfluous, so that it would be easier to determine the end of the TG experiment.

In chapter 4 we highlight the main bottleneck of the α2M-T macroglobulin correction. In the experimental procedure, the course of total amidolytic activity $f_a(t)$ is found, consisting of free thrombin $f_g(t)$ and α2M-T. Because the velocity of formation of α2M-T in the pseudo first order reaction α2M + T → α2M-T is proportional to the concentration of free thrombin, the total amount of α2M-T formed is *k* times the integral of the thrombin-time curve. It is therefore seemingly easy to estimate the contribution of α2M-T to the total amidolytic activity. In practice however there is one glitch, because in order to be able to determine this constant a point in time has to be found where all free thrombin has disappeared. The key element in this process is that if *k* is underestimated, part of the activity of α_2M-T is attributed to free thrombin and contributes to the ETP, whereas when *k* is overestimated part of the free thrombin is attributed to α_2M-T. With use of the current substrate no satisfiable solution to end level determination has been found yet.

Besides the perfect substrate, which might be challenging to synthesise, gains can already be made by altering the current substrate concentration. Lowering to substrate concentration to e.g. 150 μM would not only reduce the inner filter effect but also benefits the TG experiment as there is less competition between the substrate and the serpins inhibiting TG.

Technical ameliorations to the device.

An obvious improvement would be the level of accuracy of the temperature control within the TG device. Whatever the temperature of the measurement will be, there is still a need for accurate temperature control as measuring at room temperature is not an option. The inter-laboratory temperature differences as well as seasonal variations in the laboratory would then prove to be new sources of error. A second adaptation would be the excitation light source, the stability of the intensity of the light source is of vital importance especially when a standard correction factor for the inner filter effect is to be used. Only LED's fulfil these requirements because they are stable throughout their lifetime. Both tungsten-halogen and mercury/xenon arc lamps, the only reasonable alternatives, show a decay with time and they have considerable pre-heating times during which their output intensity is highly variable.

In short: The CAT-TG test that is the most used at the moment and, in a slightly modified form, a candidate for clinical application is essentially the same as that proposed by Hemker et al. almost twenty years ago. Hundreds of articles have shown its potential use in clinical and fundamental research and promise it to be useful in patient care. In this thesis we show that fundamental ameliorations of the original design are possible and desirable.

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