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Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram

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Summary

Elevated procoagulant levels have been correlated with increased thrombin generation *in vitro* and with increased venous thromboembolism (VTE) risk in epidemiological studies. Thrombin generation tests are increasingly being employed as a high throughput method to provide a global measure of procoagulant activity in plasma samples. The objective of this study was to distinguish the effects of assay conditions [tissue factor (TF), thrombomodulin, platelets/lipids] and factor levels on thrombin generation parameters, and determine the conditions and parameters with the highest sensitivity and specificity for detecting elevated factor levels. Thrombin generation was measured using calibrated automated thrombography (CAT) in corn trypsin inhibitor (CTI)-treated platelet-free plasma (PFP) and platelet-rich plasma (PRP). Statistical analysis was performed using logarithms of observed values with analysis of variance that accounted for experiment and treatment. The relative sensitivity of lag time (LT), time to peak (TTP), peak height and endogenous thrombin potential (ETP) to elevated factors XI, IX, VIII, X, and prothrombin was as follows: PFP initiated with 1 pM TF > PFP initiated with 5 pM TF > PRP initiated with 1 pM TF. For all conditions, inclusion of thrombomodulin prolonged the LT and decreased the peak and ETP; however, addition of thrombomodulin did not increase the ability of CAT to detect elevated levels of individual procoagulant factors. In conclusion, CAT conditions differentially affected the sensitivity of thrombin generation to elevated factor levels. Monitoring the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF was most likely to detect hypercoagulability due to increased procoagulant factor levels.

Keywords

Thrombin; tissue factor; thrombomodulin; platelets; hypercoagulability; thrombosis

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Introduction

Thrombin activates platelets, converts fibrinogen to fibrin, and initiates wound healing. Elevated procoagulant levels are correlated with abnormal thrombin generation *in vitro* (1–5) and increased risk of VTE (6–12). For example, prothrombin concentrations >115% of normal are associated with a 2.1-fold increased VTE risk, and factor VIII concentrations >200% are associated with markedly elevated VTE risk (OR = 10.8) (6,7). Tests that assess the ability of plasma to generate thrombin are thought to provide a more global measure of hemostasis and thrombosis than standard clotting assays. Such tests may be useful for determining risk of primary and recurrent VTE, and therefore, duration of antithrombotic therapy.

The most commonly used assay for continuous thrombin measurement is calibrated automated thrombography (CAT) (13). CAT is based on monitoring cleavage of a slow-reacting fluorogenic substrate and comparing it to a known thrombin concentration added to non-clotting samples (13). Studies have demonstrated CAT's ability to detect reduced thrombin generation in clotting factor deficiency (14–17). CAT appears most sensitive to hypocoagulability when initiated by low (1 pM) TF (16).

Given these findings, it is logical to hypothesise that CAT can be used to detect *hyper*coagulability due to elevated factor levels. Using high (184–429 pM) TF and no added phospholipids, Siegemund *et al.* observed increased ETP in platelet-poor plasma with high concentrations of factors XI and IX, but not VIII (4). In contrast, in PRP assays in which clotting was initiated with low (0.5 pM) TF, Regnault *et al.* observed increased ETP with elevated factor VIII (3). Several studies have correlated recurrent VTE risk with elevated peak height and/or ETP (18–24). However, another study correlated abnormal thrombin generation with first, but not recurrent VTE (25). It has been proposed that differences in findings from these studies stem, in part, from variations in assay conditions (25–27). Notably, these studies differed in the source and concentration of TF and phospholipids, and use (or not) of contact pathway inhibitors (CTI) and protein C pathway sensitising agents [activated protein C (APC), thrombomodulin] because the experimental conditions providing the highest sensitivity and specificity for detecting hypercoagulability have not been defined. The goals of this study were to:

1. distinguish the effects of assay conditions (added TF, thrombomodulin, platelets/phospholipids) and elevated factor levels on thrombin generation parameters, and
2. determine the conditions and parameters with the highest sensitivity and specificity for detecting elevated factor levels.

We simulated “hypercoagulability” by spiking plasma with factors XI, IX, VIII, X, V, prothrombin, or fibrinogen to 200% and 400% of normal, and measured the effect of these factors on thrombin generation parameters in different experimental conditions. We report that the conditions under which CAT was performed differentially affected its sensitivity. Changes in the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF was most likely to detect hypercoagulability due to increased factor levels.

Materials and methods

Materials and proteins

All proteins used in this study were of the highest quality and specific activity available. CTI and factors XI, V, and prothrombin were purchased from Haematologic Technologies, Inc (Essex Junction, VT). Factor X and plasminogen-, von Willebrand factor- and fibronectin-depleted fibrinogen were purchased from Enzyme Research Laboratories (South Bend, IN); factor X was further purified as described (5). Factor VIII (Hemofil M, Baxter, Deerfield, IL)

and factor IX were the generous gifts of Dr. Dougald M. Monroe (UNC at Chapel Hill). Briefly, factor IX was prepared from prothrombin complex concentrations by Q Sepharose chromatography, heparin affinity, metal chelate chromatography using copper, and pseudo-affinity calcium elution from HiTrap Q. Factor IX could be activated completely by factor XIa and gave the expected molar concentration when the activated material was titrated with antithrombin in the presence of heparin. Protein purity was verified by both non-reducing and reducing 12% SDS-PAGE prior to use. Analysis of factors XI, IX, X, V, prothrombin, and fibrinogen:

1. revealed only bands of the expected molecular weight for each zymogen and cofactor, and
2. indicated no activated protein species (data not shown).

The factor VIII preparation (Hemophil M) also contained albumin, however, control experiments showed no effect of this albumin concentration on thrombin generation (data not shown). The specific activity of the factor V preparation was 51.3 U/mg, consistent with that expected for factor V (50–60 U/mg), and ~40× lower than that expected for factor Va (2000 U/mg), indicating no factor Va contamination in the preparation. Rabbit lung thrombomodulin was from American Diagnostica, Inc (Stamford, CT). Fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC), TF/Lipid Reagents (PFP-Low, PFP-High), and thrombin calibrator (α2-macroglobulin/thrombin) were generously provided by Diagnostica Stago (Parsippany, NJ). Lipids were composed of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine at a ratio of 20:60:20, respectively. Blood was collected according to protocols approved by the UNC Institutional Review Board.

Plasma preparations

For PFP, blood was collected from 18 healthy individuals [20–45 years old, 50% female, 50% non-Caucasian, with normal partial thromboplastin times (PTT)] through a 21-gauge needle into a syringe and immediately transferred to sodium citrate/CTI [0.105 M (3.2%, pH 6.5) and 18.3 μg/mL, respectively] to minimise contact activation (28,29). PFP was prepared by sequential centrifugation (150 × *g*, 15 minutes, 13,000 × *g*, 15 minutes), aliquoted, and snap-frozen in liquid nitrogen within two hours of phlebotomy. PFPs were then thawed, pooled, and refrozen for subsequent assays. Pooled PFP was analyzed for levels of factors XI, IX, VIII, X, V, VII, protein C, free protein S, prothrombin, and fibrinogen at UNC Hospitals McLendon Clinical Laboratory; all were in the normal reference range.

For PRP, blood was collected from six healthy individuals (21–50 years old, 67% female, normal PTTs) who had not taken aspirin for two weeks or ibuprofen for three days prior to phlebotomy. Blood was drawn as for PFP. PRP was prepared by centrifugation (150 × *g*, 15 minutes) within one hour. The platelet count was measured on a Z1 Series Coulter Counter (Beckman Coulter, Fullerton, CA), and normalised by dilution with autologous PFP to 225,000/μl, and used at 150,000/μl (final).

Thrombin generation assay

Factors XI, IX, VIII, X, V, prothrombin or fibrinogen were added to normal, pooled PFP and individual PRPs to 200% or 400% of normal (final). Thrombin generation was measured by CAT (13). For PFP experiments, 20 μl of TF/phospholipids were mixed with 80 μl PFP in 96-well round-bottom microtiter plates (Becton Dickinson, Falcon™). The plate was inserted into a Fluoroskan Ascent® fluorometer (ThermoLabsystem, Helsinki, Finland) and warmed to 37 °C for 5 minutes. Reactions were initiated by automatically dispensing 20 μl of 2.5 mM fluorogenic substrate in 0.1 M $CaCl_2$ to each well. Reactions were performed in duplicate or triplicate in each experiment. The final TF, phospholipid, fluorogenic substrate, and $CaCl_2$

concentrations were 1 or 5 pM, 4 μM, 416 μM and 16 mM, respectively. Experiments with PRP were performed under identical conditions, but without added phospholipids. Reactions were calibrated against wells containing 20 μL of α2-macroglobulin/thrombin complex and 80 μL PFP or PRP (13). Thrombin generation was monitored at 37 °C with excitation and emission filters at 390 nm and 460 nm, respectively, every 20 seconds for 120 minutes. Experiments with PFP and 1 pM TF, PFP and 5 pM TF, and PRP and 1 pM TF were performed 10, 5, and 6 times, respectively.

Thrombin parameters were calculated using Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands), which defines the LT as the first time point after the thrombin concentration exceeds one-sixth peak height, the TTP as the time to the peak height, the peak height as the maximum thrombin concentration produced, and the ETP as the time integral of thrombin formation (the area under the thrombin generation curve).

Statistical methods

Statistical analysis was performed for logarithms of parameters using analysis of variance (ANOVA) that accounted for experiment date (PFP experiments), or donor (PRP experiments) and elevated factors. Each condition (factor level) was handled separately; factor concentration was not considered a covariate. An ANOVA model was first fit to each log-transformed parameter with covariates of date or donor, factor level, an indicator variable for platelets, and the interaction between factor level and platelet indicator. We compared PFP initiated with 1 pM TF to:

1. PFP initiated with 5 pM TF, and
2. PRP initiated with 1 pM TF.

Hypothesis testing was performed using a contrast matrix corresponding to a 1 degree of freedom F-test for each parameter. In a separate analysis, for each log-transformed parameter, a separate ANOVA model was fit with covariates of date or donor and factor level. To determine the effect of thrombomodulin in the presence of elevated factors, ANOVA models were applied to the log of the ratio of the parameter value with and without thrombomodulin. Differences in least square means between conditions and the control were evaluated by t-tests. Multiple comparisons were handled with Bonferroni corrections; significance was $p<0.0033$ (0.05/15) unless otherwise indicated. The percent coefficient of variation (%CV) was calculated as the ratio of the standard deviation to the mean multiplied by one hundred for inter-assay variability (PFP) and inter-individual variability (PRP).

Results

Thrombin generation in normal PFP and PRP

We first characterised the effects of TF on thrombin generation in normal PFP and PRP. Thrombin generation in PFP required addition of both TF and phospholipids; omission of either prevented thrombin generation for >120 minutes (data not shown), indicating that the PFP was not contaminated with detectable endogenous TF activity, procoagulant lipids, or contact activation. Similarly, thrombin generation in PRP required addition of TF (data not shown), indicating that PRP from these healthy subjects did not contain detectable TF activity or contact activation.

Table 1 shows baseline thrombin generation characteristics for PFP and PRP. Reactions initiated by 1 and 5 pM TF are indicated: $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$, and $PRP_{(1\ pM\ TF)}$, respectively. Compared to $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$ demonstrated shortened LT and TTP and increased peak height and ETP. The inter-assay variability (%CV) of PFP (Table 2) and inter-individual variability (%CV) in PRP (Table 3) were consistent with prior studies (13,26,30).

Thrombin generation in $PFP_{(1\ pM\ TF)}$ demonstrated a significantly shorter LT and TTP, increased peak height and decreased ETP than $PRP_{(1\ pM\ TF)}$, consistent with previous studies (13,26,30).

Effect of thrombomodulin on thrombin generation in normal PFP and PRP

Thrombin generation in normal PFP is decreased in the presence of thrombomodulin or APC (3,13,22,24,31,32); however, patients with a protein C pathway deficiency (factor V Leiden, protein C/S deficiency) have decreased thrombomodulin/APC sensitivity (22,24). Previous studies have recommended that CAT assays include APC or thrombomodulin to increase ability to discriminate between healthy subjects and VTE patients, as well as predict VTE recurrence (19,23,24,31). We first compared thrombin generation in the presence and absence of thrombomodulin in $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$ (Fig. 1a, 1b, Table 1). Thrombomodulin slightly prolonged the LT and reduced the peak height and ETP in both $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$. Thrombomodulin slightly shortened the TTP in $PFP_{(1\ pM\ TF)}$, but not $PFP_{(5\ pM\ TF)}$. Increased TF decreased thrombomodulin sensitivity; that is, 5 nM thrombomodulin reduced the ETP ~87±1.9% and ~28±3.1% for $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$, respectively. Thrombomodulin significantly increased the inter-assay %CV of the peak height and ETP in $PFP_{(1\ pM\ TF)}$, but had less effect in $PFP_{(5\ pM\ TF)}$ (Table 2).

We then examined the impact of thrombomodulin on thrombin generation in $PRP_{(1\ pM\ TF)}$. As in $PFP_{(1\ pM\ TF)}$, thrombomodulin prolonged the LT and reduced the peak height and ETP in $PRP_{(1\ pM\ TF)}$ (Fig. 1c, Table 1). In contrast to $PFP_{(1\ pM\ TF)}$, thrombomodulin slightly prolonged the TTP in $PRP_{(1\ pM\ TF)}$, likely because the TTP in PRP is limited by the time to platelet activation. These findings are somewhat different, although not altogether inconsistent with previous findings that thrombomodulin decreased ETP, but did not change LT or TTP in PRP (13). The subtle differences in response to thrombomodulin in these studies may relate to the thrombomodulin source (human *vs.* rabbit lung), although another study found similar effects of thrombomodulin from different sources on ETP (24). The inter-individual variability increased for LT and TTP, decreased for ETP, and did not change for peak height in the presence of thrombomodulin (Table 3). Platelets decreased CAT sensitivity to thrombomodulin; that is, 5 nM thrombomodulin reduced the ETP ~66±4.8% in $PRP_{(1\ pM\ TF)}$, compared to ~87±1.9% in $PFP_{(1\ pM\ TF)}$ (Fig. 1c,Table 1). The reduced thrombomodulin sensitivity of PRP *versus* PFP likely reflects contributions of platelet-derived factor V(a) to thrombin generation (33,34), and is consistent with previous studies (13).

Effect of procoagulant factors on thrombin generation in PFP and PRP

Finally, we compared the ability to detect hypercoagulability under each of these conditions (1 and 5 pM TF, in the presence and absence of thrombomodulin, in PFP and PRP). We measured thrombin generation in PFP and PRP spiked with factors XI, X, IX, VIII, V, prothrombin, or fibrinogen. We initiated clotting in PFP with 1 or 5 pM TF and 4 μM phospholipids, and in PRP with 1 pM TF (no phospholipids), in the presence and absence of thrombomodulin.

LT

The LT in $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$ were qualitatively similar, although changes in $PFP_{(5\ pM\ TF)}$ were smaller than changes in $PFP_{(1\ pM\ TF)}$ (Fig. 2a, 2b). In both the absence and presence of thrombomodulin, intrinsic factors XI, IX, and VIII did not significantly alter the LT, although effects were slightly greater in the presence of thrombomodulin. Factor X significantly shortened the LT in both conditions. Interestingly, factor V significantly shortened the LT in $PFP_{(1\ pM\ TF)}$ but not $PFP_{(5\ pM\ TF)}$. Both prothrombin and fibrinogen significantly increased the LT, irrespective of the TF concentration or presence of TM. The opposing effects of factor X and prothrombin on LT in PFP may relate to differences in their plasma

concentrations. Whereas 200% factor X increases the plasma level from 8 to 16 μg/mL, 200% prothrombin increases the plasma level from 100 to 200 μg/mL. This high concentration of prothrombin or the prothrombin activation product fragment 1.2 may competitively inhibit binding of other Gla proteins to the lipid surface, thereby prolonging the time to complex assembly and enzyme generation. Indeed, elevated prothrombin did not prolong the LT in assays performed in the presence of increased (300 μM) lipid concentrations (data not shown).

Procoagulant factors produced unique effects on the LT in $PRP_{(1\ pM\ TF)}$ (Fig. 2c). In both the presence and absence of thrombomodulin, factors XI, IX, VIII, X, prothrombin, and fibrinogen non-significantly shortened the LT in $PRP_{(1\ pM\ TF)}$; only 400% factor XI significantly shortened the LT. These findings suggest that in contrast to PFP, the LT in PRP is predominated by the time required for platelet activation and supercedes the time necessary to form procoagulant complexes on exposed lipid. Factor V exhibited a biphasic effect on LT in $PRP_{(1\ pM\ TF)}$, significantly increasing the LT at 200%, with no effect at 400%. These interesting observations were unique to experiments with platelets and may reflect differences in the procoagulant activity of plasma and platelet-derived factor V(a) (34).

TTP

Factors XI, IX, and VIII generally shortened the TTP in $PFP_{(1\ pM\ TF)}$, but did not affect the TTP in $PFP_{(5\ pM\ TF)}$ (Fig. 3a, 3b). Factor X shortened the TTP in both $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$. Factor V and fibrinogen produced little to no effect on TTP in either condition. With the exception of prothrombin, the factors produced qualitatively similar effects in $PRP_{(1\ pM\ TF)}$ as in $PFP_{(1\ pM\ TF)}$, with a slight decrease in the TTP with factors XI, IX, VIII, X, and fibrinogen (Fig. 3c). Effects were similar in the presence and absence of thrombomodulin.

Interestingly, as seen in the LT, whereas prothrombin prolonged the TTP in PFP, it shortened the TTP in PRP. Again, prothrombin's effect may arise in PFP because of prothrombin binding to the constitutively exposed lipid surface of the synthetic lipids, whereas the lipid surface is protected from prothrombin binding on unactivated platelets until the time of activation. Platelet activation permits simultaneous formation of both tenase and prothrombinase, preventing inhibition by high prothrombin concentrations.

Peak height

In contrast to effects on LT and TTP, factors exhibited similar effects on peak height in PFP and PRP; however the magnitude of the effects differed substantially (Fig. 4a-4c). For all conditions, factors XI, IX, VIII, X, prothrombin, and fibrinogen increased the peak height, whereas factor V had little effect on peak height. Changes were largest in $PFP_{(1\ pM\ TF)}$, followed by $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$, respectively, but similar in the presence and absence of thrombomodulin for all three conditions.

ETP

As with peak height, factors similarly affected ETP in PFP and PRP; however, the magnitude of the effects differed substantially with the different conditions (Fig. 5a-5c). Factors IX, VIII, X, prothrombin, and fibrinogen increased the ETP in $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$, and $PRP_{(1\ pM\ TF)}$. Factor XI increased ETP only in reactions initiated with 1 pM TF, in the presence of thrombomodulin. Factor V had little effect on ETP in any condition.

Discussion

Using a range of TF and phospholipid concentrations in the presence and absence of protein C pathway inhibitors, previous studies have correlated VTE risk with elevated peak height (18), ETP (20,22–25) or both (19,21). Other studies have focused on the LT, rate, and peak as

an indicator of circulating TF and prothrombotic disease (19,35,36). Although differences in findings between studies have been attributed to differences in assay conditions, few studies have contrasted different assay conditions within a single design, and even fewer under *hyper*coagulable conditions. It has not been explicitly shown that all conditions provide equal sensitivity *and* specificity to detect hypercoagulability. Given limited plasma sample sizes and limited availability of samples from large-scale epidemiologic studies of hypercoagulability, empirical evidence for the use of specific conditions and parameters is essential for designing prospective studies of plasma hypercoagulability. Our study design permitted the *direct* comparison of conditions and parameters to identify those most sensitive and specific to factor-induced hypercoagulability. To our knowledge, this work is the first comprehensive, systematic comparison of these conditions in a single study.

Our analysis shows that in PFP, factors XI, IX, VIII, X, prothrombin, and fibrinogen significantly increased peak height and ETP, though increases were proportionally larger for peak height than ETP. Whereas the baseline peak height of $PFP_{(5\ pM\ TF)}$ was 74% higher than that of $PFP_{(1\ pM\ TF)}$ (344 nM *vs.* 197 nM, respectively, Table 1), the apparent "maximum peak height" in $PFP_{(5\ pM\ TF)}$ plus elevated factors XI, IX, VIII, X, or V up to 400% was only 23% higher than that in $PFP_{(1\ pM\ TF)}$ plus elevated factors (396 nM *vs.* 322 nM, respectively). These findings suggest that the maximum observable difference between peak height in normal and "hypercoagulable" PFP was reduced in assays initiated with higher TF (15% for $PFP_{(5\ pM\ TF)}$ *vs.* 63% for $PFP_{(1\ pM\ TF)}$. Similarly, the maximum ETP in $PFP_{(5\ pM\ TF)}$ with elevated factors XI, IX, VIII, X, or V (1925 nM*min) was only 3.8% higher than that in $PFP_{(1\ pM\ TF)}$ (1854 nM*min). Thus, the maximum observable difference in ETP between PFP and "hypercoagulable" PFP was reduced in $PFP_{(5\ pM\ TF)}$ (9.7%) *versus* $PFP_{(1\ pM\ TF)}$ (18%). This difference is even smaller than that seen for peak height. The exception to these results is in PFP containing elevated prothrombin. Prothrombin (200%) significantly increased peak height and ETP in both in both low (1 pM) (1.3-fold and 1.6-fold, respectively) and high (5 pM) (1.4-fold and 1.7-fold, respectively) TF. These findings suggest that the mechanism limiting thrombin generation in this system results, at least in part, from the prothrombin concentration and indicates that peak height and ETP are exquisitely sensitive to hyperprothrombinemia.

It is widely accepted that PRP may provide greater physiologic relevance in assays of this nature, however studies of PFP persist, in part because of logistical limitations associated with the use of fresh PRP. Our study demonstrates that CAT assays of PFP and PRP show different trends in LT and TTP, but provide qualitatively similar data on peak height and ETP. Thus, studies comparing the presence and absence of platelets are likely to provide consistent information on peak height and ETP, but inconsistent findings on LT and TTP. Differences in response of LT or TTP in PFP and PRP may, however, provide important mechanistic information on the role of platelets and platelet abnormalities in thrombosis.

Epidemiologic studies have correlated elevated levels of factors XI, IX, VIII, X, prothrombin, or fibrinogen, but not factor V, with increased VTE risk (6–12). In this regard, CAT appears specific to hypercoagulability due to these factors. It is important to note, however, that the magnitude of change in thrombin generation did not correlate with the degree of thrombotic risk associated with each factor. For example, elevated prothrombin produced a large linear increase in peak height. However, thrombotic risk associated with elevated prothrombin is relatively small (OR ~2.1) (7). Discrepancies between the thrombin generation measurements and clinical risk in the case of elevated prothrombin may result from difficulties in measuring (pro)thrombin concentrations in excess of the antithrombin level present in the plasma. Additionally, although 200% fibrinogen increased the peak height and ETP, its role in VTE risk is controversial. It is unclear whether the effects of fibrinogen on thrombin generation parameters resulted from changes in thrombin generation, itself, or in the ability of fibrinogen

("antithrombin I") to bind thrombin and preserve its proteolytic activity towards small molecular substrates. Thus, CAT may demonstrate disproportionately high peak height and ETP for patients with lower overall risk, reducing the specificity and predictive value of this technique in certain patients.

As a means of distinguishing effects of procoagulant factors on CAT, we note that whereas several clotting factors significantly increased peak height and ETP, these factors had different and sometimes unique effects on other parameters. For example, in $PFP_{(1\ pM\ TF)}$, elevated levels of factors IX, X, and prothrombin significantly increased peak height and ETP. However, factor IX did not affect the LT, factor X significantly decreased the LT, and prothrombin prolonged the LT in low but not high lipid concentrations (data not shown). Thus, analysis of multiple experimental conditions (*e.g.*, varied lipid concentrations) and/or parameters may be helpful in discerning factors contributing to abnormal thrombin generation in certain PFPs. Indeed, Tripodi *et al.* recently suggested that use of three abnormal thrombin generation parameters (LT, peak height, and ETP) improves the identification of patients at risk of recurrent VTE *versus* analysis based on a single parameter (37).

Comparisons of findings between centers have been difficult, as inter-center variability is high partly due to the use of "in house" reagents and protocols. Using a particularly elegant study design, Dargaud *et al.* (2007) showed that the use of different TF and phospholipid sources produces large variability in CAT, but standardising conditions significantly reduces center-to-center variability (27). We used commercially-available reagents from the CAT manufacturer, which may offer consistency in results and enable the continued evaluation of these conditions in future studies.

This study has several limitations. First, although thrombomodulin may be helpful in characterising plasmas with proteins C or S deficiency or factor V Leiden, the physiological thrombomodulin concentration has not been established because it is primarily a cell-associated protein. Thus, the concentration of soluble thrombomodulin that provides the most clinically-useful information has not been determined. Our data suggested that thrombomodulin increased the inter-assay %CV and dampened the effects of 200% fibrinogen at low TF, but did not significantly impact the ability to detect hypercoagulability from the other factors tested. Thus, its general use for detecting protein C pathway abnormalities is compatible with assays used to detect elevated levels of clotting factors. Second, although epidemiological studies have suggested elevated levels of certain clotting factors independently increase VTE risk (6–12), the factor levels we tested were generally higher than those reported in these studies. Of note, however, prothrombin levels as high as 500% of normal have been reported in patients with type 2 diabetes (38). CAT's ability to detect elevated prothrombin suggests abnormal thrombin generation in these patients would be readily detected by this technique. Third, our study design compared frozen/thawed, pooled PFP with individual PRP, which may emphasise differences in %CV between PFP and PRP. Fourth, as opposed to tests of individual factor levels, CAT offers the advantage of testing global haemostatic potential. Effects of multiple abnormal coagulation factor levels on thrombin generation may be additive, synergistic, or reflect only the effects of the most limiting factor. Additional studies are warranted to fully appreciate the effects of multiple factor abnormalities on thrombin generation measured with this technique. Finally, it is not clear whether CAT recapitulates the *in vivo* pathologic effects of procoagulant factors; other mechanisms besides thrombin generation may contribute to thrombotic risk.

In sum, we have shown that CAT's ability to detect elevated factors varies between factors and depends on the assay conditions. The largest changes in thrombin generation in response to elevated factors XI, IX, VIII, X, or prothrombin were seen in peak height and ETP in $PFP_{(1\ pM\ TF)}$. Smaller changes were observed in $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$. Therefore,

monitoring the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF is most likely to detect hypercoagulability due to increased procoagulant factor levels.

What is known about this topic?

Thrombin generation tests can detect hypocoagulability. Significant interest lies in determining their ability to detect hypercoagulability.

Previous studies have correlated primary or recurrent VTE risk with abnormal thrombin generation; however, the experimental conditions differ significantly in these studies.

Standardisation of thrombin generation tests reduces the variability of results and is necessary for its continued clinical development.

The experimental conditions providing the highest sensitivity and specificity for detecting hypercoagulability have not been identified.

What does this paper add?

The ability to detect elevated factors varies between factors and depends on the assay conditions.

The relative sensitivity of lag time (LT), time to peak (TTP), peak height and endogenous thrombin potential (ETP) to elevated factors XI, IX, VIII, X, and prothrombin was as follows: PFP initiated with 1 pM TF > PFP initiated with 5 pM TF > PRP initiated with 1 pM TF.

Monitoring the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF is most likely to detect hypercoagulability due to increased procoagulant factor levels.

Our findings support efforts to standardise reagents (TF and lipid concentrations) (27) to reliably achieve the assay conditions necessary for maximal sensitivity. Our data also confirm effects previously seen in normal plasma, but importantly, extend these findings to hypercoagulable situations. Identification of conditions that best identify hypercoagulability and predict VTE warrants further investigation. *In vivo*, VTE risk likely depends on a combination of increased procoagulant and decreased anticoagulant activities, and/or other pathologic mechanisms. In a clinical setting, the concerted use of several different assay conditions may be necessary to identify patients with distinct clinical phenotypes, not unlike the use of both aPTT and PT to diagnose factor deficiencies.

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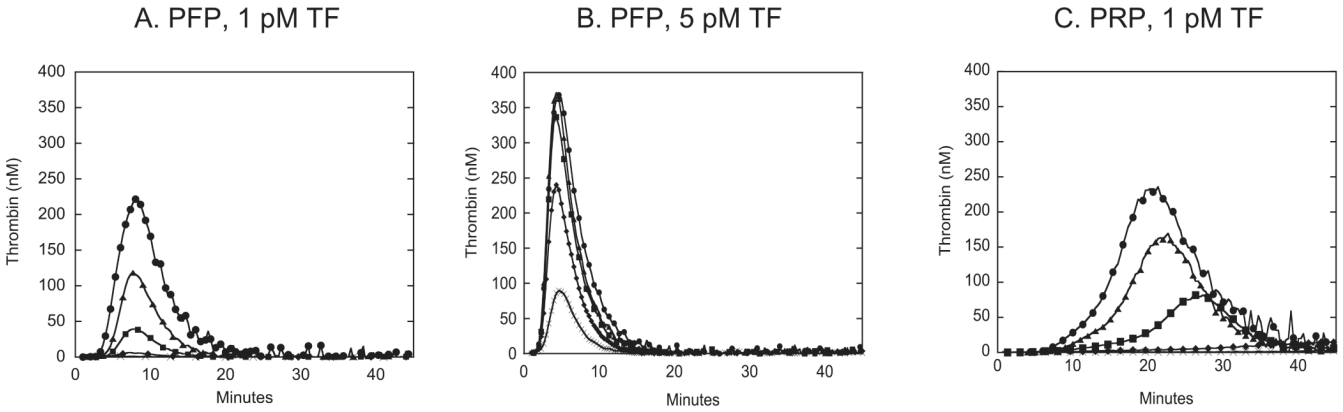

Figure 1. Thrombomodulin decreases thrombin generation in $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$

A, B) Thrombin generation in PFP was initiated by addition of 4 μM phospholipids and A) 1 pM TF or B) 5 pM TF. C) Thrombin generation in PRP was initiated by addition of 1 pM TF. Curves show increasing thrombomodulin: 0 (circles), 2.5 (triangles), 5 (squares), 10 (diamonds), and 20 (crosses) nM, representative of 3 separate experiments.

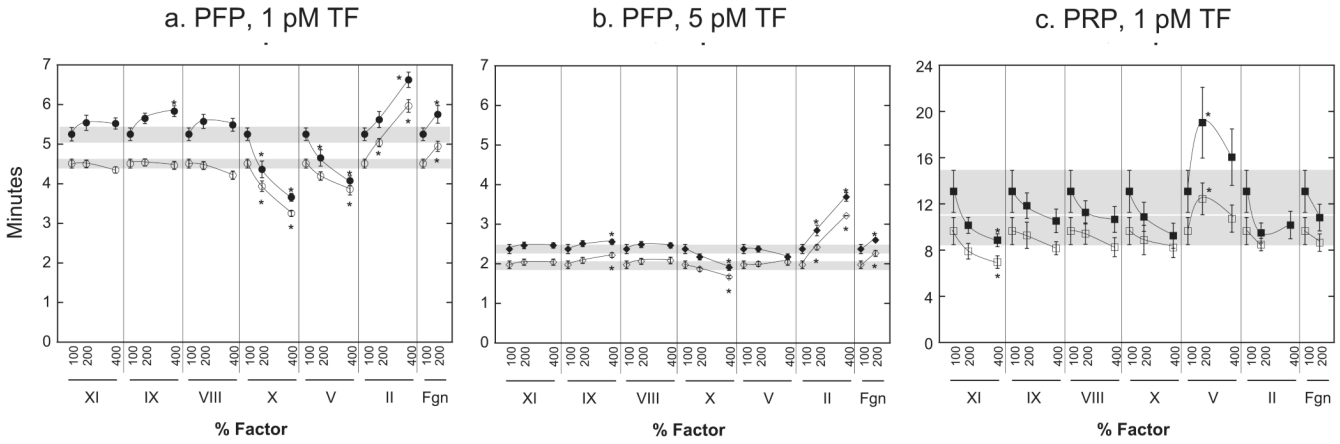

Figure 2. Procoagulant factors and thrombomodulin differently influence LT in PFP and PRP

PFP or PRP was spiked with factors XI, IX, VIII, X, V, prothrombin, or fibrinogen to 200% or 400% of normal. Clotting was initiated by addition of 1 pM TF and 4 μM phospholipids (circles), 5 pM TF and 4 μM phospholipids (diamonds), or 1 pM TF (no lipids, squares) for A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, and C) $PRP_{(1\ pM\ TF)}$, respectively, in the absence (open symbols) and presence (closed symbols) of thrombomodulin, as described in methods. Note y-axis scaling in panel C. The shaded gray box encompasses the 100% value and its standard error of the mean (SEM). Data show means ±SEM. *p<0.0033 *versus* 100%.

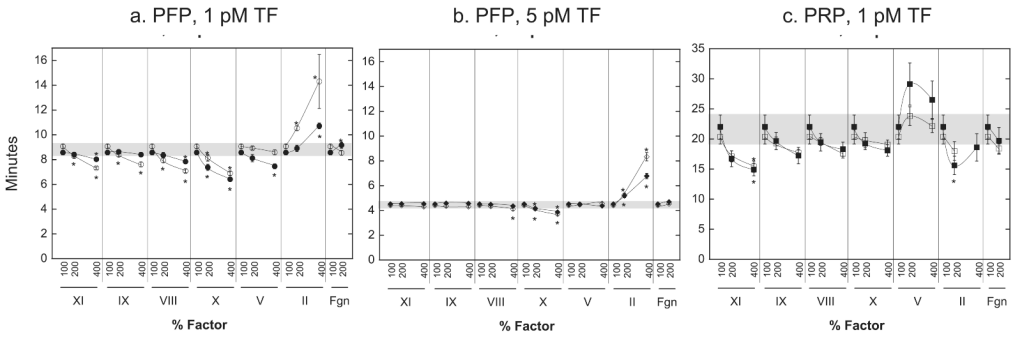

Figure 3. Procoagulant factors and thrombomodulin differently influence TTP in PFP and PRP

Reactions were performed as in Fig 2. A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note y-axis scaling in panel C. *p<0.0033 *versus* 100%

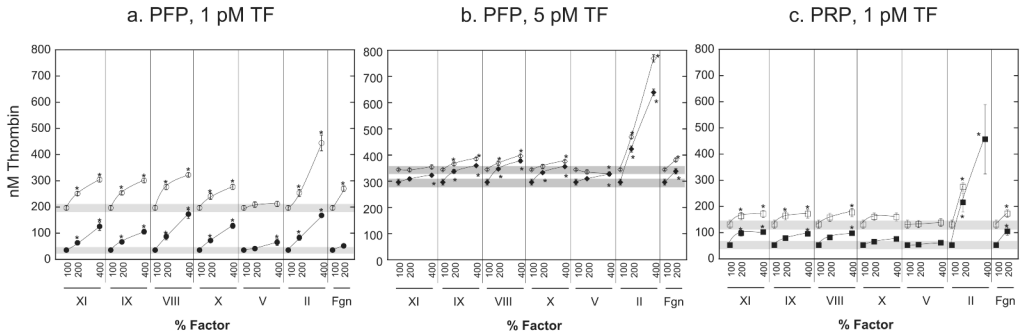

Figure 4. Procoagulant factors and thrombomodulin differently influence peak height in PFP and PRP

Reactions were performed as in Fig 2. A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note the y-axis scaling. *p<0.0033 *versus* 100%

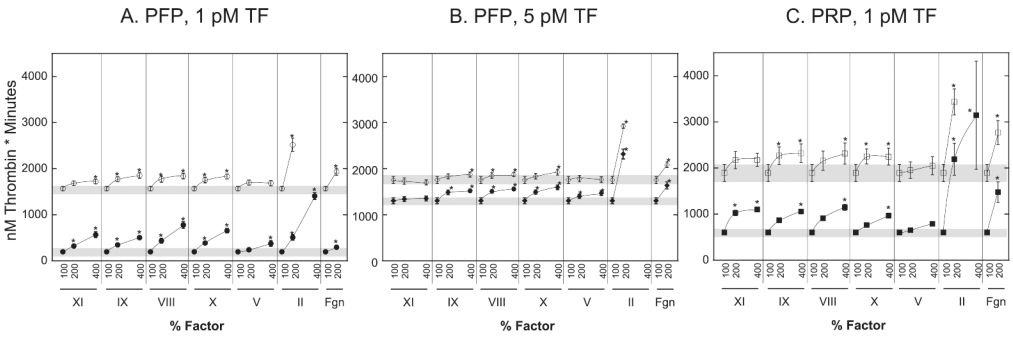

Figure 5. Procoagulant factors and thrombomodulin differently influence ETP in PFP and PRP

Reactions were performed as in Fig 2. A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note the y-axis scaling. *p<0.0033 *versus* 100%

Table 1

Baseline characteristics (±SD) of thrombin generation in PFP and PRP, in the absence and presence of 5 pM thrombomodulin (TM)

	PFP$_{(1\ pM\ TF)}$				PFP$_{(5\ pM\ TF)}$				PRP$_{(1\ pM\ TF)}$			
	-TM		+TM		-TM		+TM		-TM		+TM	
LT (min)	4.5	± 0.4	5.3	± 0.5*	2.0	± 0.2^	2.4	± 0.2*	9.7	± 2.9^	13.1	± 4.4*
TTP (min)	9.1	± 0.6	8.6	± 0.6*	4.4	± 0.3^	4.5	± 0.2	20.4	± 2.9^	22.0	± 4.8
Peak height (nM)	197.0	± 30.2	35.9	± 16.8	344.4	± 10.4^	296.4	± 22.2*	132.2	± 37.6^	52.6	± 15.6*
ETP (nM.min)	1569.1	± 168.8	197.9	± 90.2*	1754.7	± 176.2^	1303.9	± 128.7*	1893.6	± 455.3^	602.1	± 94.9*

*$p<0.0001$ *vs.*"-TM".

^$p<0.0001$ *vs.* "PFP$_{(I\ pM\ TF)}$-TM".

Table 2
Inter-assay variability (%CV) of thrombin generation in PFP

	$PFP_{(1\ pM\ TF)}$		$PFP_{(5\ pM\ TF)}$	
	-TM	**+TM**	**-TM**	**+TM**
LT	8.4	9.2	10.8	9.8
TTP	6.4	7.0	5.8	5.3
Peak height	15.4	46.8	3.0	7.5
ETP	10.8	45.6	10.0	9.9

Table 3
Inter-individual variability (%CV) of thrombin generation in PRP

	$PRP_{(1\ pM\ TF)}$	
	-TM	**+TM**
LT	29.7	33.9
TTP	14.4	21.9
Peak height	28.4	29.7
ETP	24.0	15.8